HISTOPATHOLOGICAL AND IMMUNOHISTOCHEMICAL STUDY OF HEPATOBLASTOMA

A DISSERTATION SUBMITTED IN PARTIAL FULFILMENT OF THE

REQUIREMENTS FOR THE AWARD OF THE MD BRANCH III

(PATHOLOGY) DEGREE EXAMINATION OF THE TAMIL NADU

DR.M.G.R MEDICAL UNIVERSITY, CHENNAI, TO BE HELD IN

APRIL 2016.

DECLARATION

This is to declare that the dissertation entitled **"Histopathological and immunohistochemical study of Hepatoblastoma"** is my original work done under the guidance and supervision of Dr. Banumathi Ramakrishna, MBBS, MD, MAMS, Professor & Head, Department of Pathology, Christian Medical College, Vellore, in partial fulfillment of the M.D. Branch III (Pathology) Degree Examination of the Tamil Nadu Dr. M.G.R. Medical University, Chennai, to be held in April 2016. I have independently reviewed the literature, standardized the data collection methodology and carried out the evaluation towards completion of the dissertation.

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CERTIFICATE

This is to certify that the dissertation entitled **"Histopathological and immunohistochemical study of Hepatoblastoma"** submitted by Dr. Kiruthiga K.G., in partial fulfilment of the requirement for the M.D. Branch III (Pathology) Degree Examination of the Tamil Nadu Dr. M.G.R. Medical University, Chennai, to be held in April 2016, is the bonafide work done by her under my guidance.

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Sub: **Fluid Research Grant Project:**
Hepatoblastoma: A Histopathological and Immunohistochemical study.
Dr. Kiruthiga.K. G, General Pathology, Dr. Banumathi Ramakrishna, Pathology, CMC, Vellore.

Ref: IRB Min No: 8985 [OBSERVE] dated 04.08.2014

Dear Dr. Kiruthiga.K. G ,

The Institutional Review Board (Blue, Research and Ethics Committee) of the Christian Medical College, Vellore, reviewed and discussed your project entitled "Hepatoblastoma: A Histopathological and Immunohistochemical study." on August 4th 2014.

The Committees reviewed the following documents:

1. IRB Application format
2. Curriculum Vitae' of Drs. Kiruthiga. K. G, Banumathi Ramakrishna
3. Proforma
4. No of documents 1-3

The following Institutional Review Board (Blue, Research & Ethics Committee) members were present at the meeting held on August 4th 2014 in the CREST/SACN Conference Room, Christian Medical College, Bagayam, Vellore 632002.

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We approve the project to be conducted as presented.

The Institutional Ethics Committee expects to be informed about the progress of the project, any **adverse events** occurring in the course of the project, any **amendments in the protocol and the patient information / informed consent**. On completion of the study you are expected to submit a copy of the **final report**. Respective forms can be downloaded from the following link: http://172.16.11.136/Research/IRB_Polices.html in the CMC Intranet and in the CMC website link address: http://www.cmch-vellore.edu/static/research/Index.html.

A sum of 1,00,000/- INR (Rupees One Lakh Only) will be granted for 2 years. 50,000/- INR (Rupees Fifty Thousand only) will be granted for 12 months as an 1st Installment. The rest of the 50,000/- INR (Rupees Fifty Thousand only) each will be released at the end of the first year as 2 nd Installment following the receipt of the Interim progress/ Annual report and subsequent submission of it to the IRB.

Yours sincerely

Dr. Nihal Thomas
Secretary (Ethics Committee)
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Mr. Denzil. S,
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Dear Mr. Denzil,

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The Institutional Review Board at its meeting held on August 4th 2014 vide IRB Min. No. 8985 accepted the project for a sum of *1,00,000/- INR (Rupees One Lakh Only) will be granted for 2 years. 50,000/- INR (Rupees Fifty Thousand only) will be granted for 12 months as an Ist Installment. The rest of the 50,000/- INR (Rupees Fifty Thousand only) each will be released at the end of the first year as 2 nd Installment following the receipt of the Interim progress/ Annual report and subsequent submission of it to the IRB.* Kindly arrange to transfer the sanctioned amount to a separate account to be operated Dr. Kiruthiga.K. G and Dr. Banumathi Ramakrishna.

Yours sincerely,

Dr. Nihal Thomas
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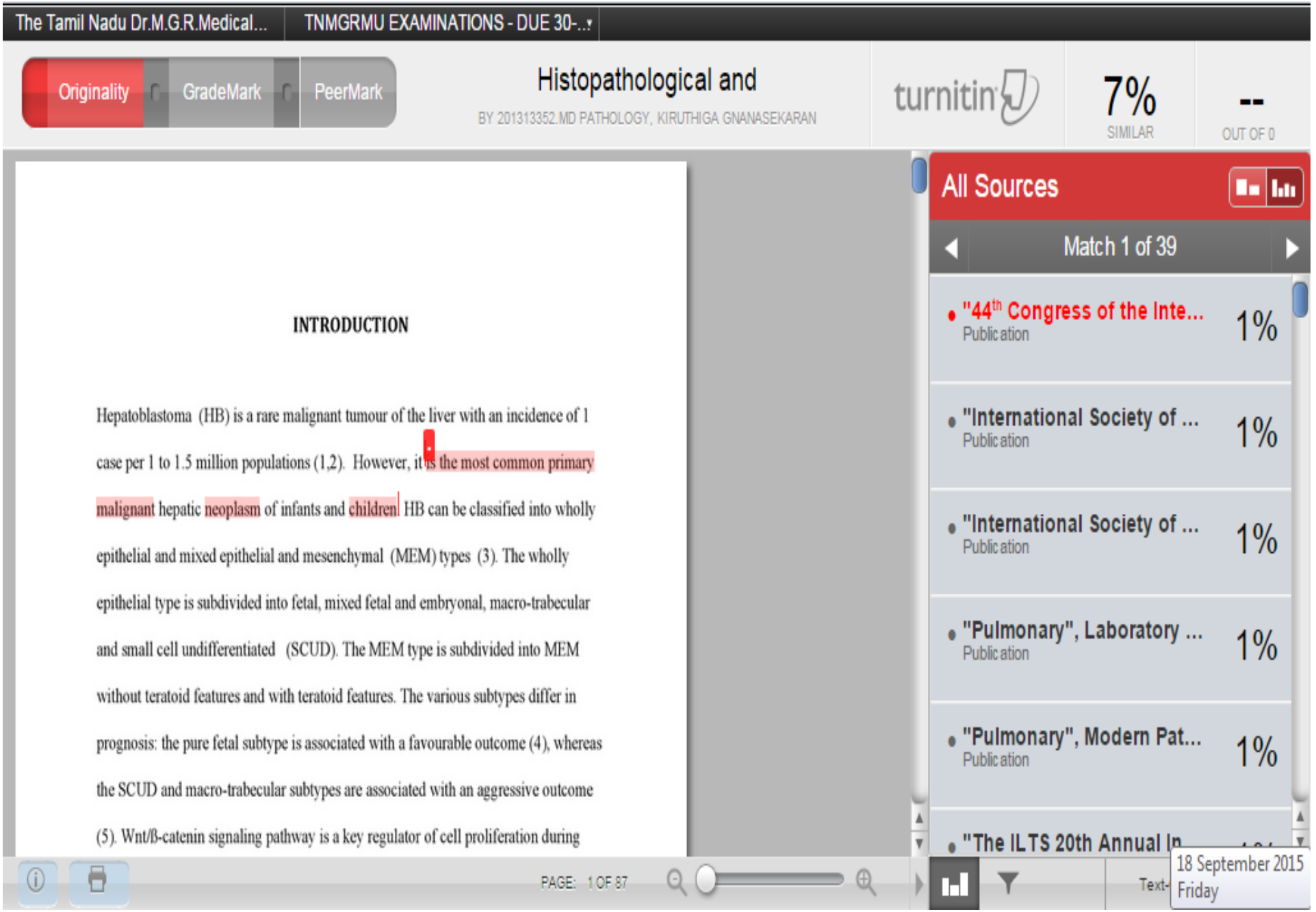
INTRODUCTION

Hepatoblastoma (HB) is a rare malignant tumour of the liver with an incidence of 1 case per 1 to 1.5 million populations (1,2). However, it is the most common primary malignant hepatic neoplasm of infants and children. HB can be classified into wholly epithelial and mixed epithelial and mesenchymal (MEM) types (3). The wholly epithelial type is subdivided into fetal, mixed fetal and embryonal, macro-trabecular and small cell undifferentiated (SCUD). The MEM type is subdivided into MEM without teratoid features and with teratoid features. The various subtypes differ in prognosis: the pure fetal subtype is associated with a favourable outcome (4), whereas the SCUD and macro-trabecular subtypes are associated with an aggressive outcome (5). Wnt/ß-catenin signaling pathway is a key regulator of cell proliferation during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previous cisplatin based chemotherapy and thus can be used as a tumour marker. It also represents a possible target for immunotherapy (10).With this background, the current study aims to morphologically classify HB cases and to correlate the expression and staining pattern of IHC markers and to determine the biological behaviour of these tumours. The expression of three markers(CK19, beta-catenin and EpCAM) will be correlated with histological subtypes, the histological parameters of tumour behaviour, response of the tumour to chemotherapy and survival.

AIMS AND OBJECTIVES

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I) To study the histopathological features of Hepatoblastoma in detail and classify them into various subtypes.

II) To identify and compare the expression of immunohistochemical (IHC) markers CK-19, EpCAM and Beta-Catenin in various subtypes of Hepatoblastoma.

III) To correlate the expression of these markers with the histological parameters of tumour behaviour and survival.

JUSTIFICATION FOR THIS STUDY

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Hepatoblastoma is the most common primary tumour of liver in children. There are different histological subtypes in HB, each with a varying prognosis. Though the survival of HB patients has shown a drastic improvement in the recent years, subsets of patients are not responsive to therapy and have a poor outcome. Therefore, it becomes important to determine and establish the factors which play an important role in the behaviour of the tumour subtypes and their correlation with the survival. There are no detailed histopathological and immunohistochemical studies on HB, comparing the subtypes and expression of various biomarkers with tumour behaviour and prognosis. This study was aimed to look at these associations.

REVIEW OF LITERATURE

REVIEW OF LITERATURE

Hepatoblastoma (HB) is the most common primary tumour of liver in children and the third most common intra-abdominal malignancy, next to Wilm's tumour and Neuroblastoma. It accounts for 25% of all hepatic tumours and 50% of malignant hepatic tumours in the pediatric population (3). The annual incidence in the Western countries is approximately 1.2-1.5 cases per million population (2,11). HB affects infants and children with around two-thirds of cases presenting in the first two years, while 90% occur within first five years of life (12,13). Only about 3% of these tumours are encountered in patients above 15 years of age (14). Wang et al, based on an extensive literature search have found about 40 cases of adult HB, with a median age of 41.5 years and associated with a grave outcome (15).

Clinical features:

The most common presentation of HB is an asymptomatic abdominal mass, while some patients complain of abdominal pain, loss of weight and emesis (16). Features like obstructive jaundice are seen in 5% of patients (14). Distant metastasis is seen in 20% of patients at the initial time of presentation and the most common site of distant metastasis is the lung(2). However rare sites of metastasis to the choroid, brain and heart have been reported(7,17).

Hepatoblastoma, although sporadic with an unknown etiology, has been found to be associated with Beckwith Wiedemann syndrome, Familial Adenomatous Polyposis, Trisomy 13, Trisomy 18, prematurity and low birth weight(18-20).

Laboratory parameters:

Serum levels of alpha feto-protein (AFP) is a very sensitive marker and is elevated in 90% of patients with HB (2). It is an excellent marker used not only in the initial diagnosis but also to monitor the response of the tumour to chemotherapy and early detection of recurrence and metastasis(21). A favoured method to monitor patients for recurrence/relapse of the disease is the serial estimation of AFP levels as compared to the computed tomographic imaging(22).

Imaging:

Imaging, as in any other tumour is useful not only for the diagnosis, but to determine the exact localisation, focality, extent and detection of metastasis. Ultrasonogram (USG) of the abdomen is the best initial mode of diagnosis of HB. By USG, around 60-70% of the tumours are localised to the right lobe of liver, although the reasons for the same are poorly understood (23). Computed tomography plays an important role in localising the tumour, detecting multi-focality and in the early detection of recurrent tumour. Imaging also finds a role in aiding the clinician to perform an image guided core needle biopsy of the tumour. Although laboratory parameters and imaging aid in the diagnosis, histopathological examination of the tumour is confirmatory and is considered gold standard (24).

Origin and Histological classification:

HB is an embryonal tumour which has been postulated to arise from the hepatic progenitor cells or the small epithelial cells (SEC). The SEC are primitive in nature that possess the property to multiply and differentiate along different lineages. These

cells have been characterized by Ruck et al on electron microscopy as ovoid cells of 7-18micrometer diameter with an electron dense scanty cytoplasm, dispersion of chromatin and a tight junction between adjacent cells. SEC exhibits a property of bidirectional differentiation into the hepatocytes and the cholangiocytes. This is exemplified by the fact that they are immunohistochemically reactive for CK-7 (biliary differentiation) as well as albumin (hepatocytic differentiation) (25). The malignant transformation of these cells and subsequent arrest in the maturation during the process of development is responsible for the diverse histological patterns seen in this tumour. Thus the various subtypes of HB correspond to a specific stage in the embryogenesis of liver depending on the stage at which the arrest has occurred.

Classification of hepatoblastoma:

Pathological classification of HB is important because it provides useful information in managing the patient and individualising the treatment. It is also important to determine and establish the factors which play an important role in the behaviour of the tumour subtype. The most widely used system of classification of HB is the one devised by Ishak and Gluntz in 1967 who classified HB into two types – pure epithelial and mixed epithelial and mesenchymal (26). Stocker, in 1994 classified HB into six major histological groups : pure fetal epithelial, mixed embryonal and fetal epithelial, macrotrabecular, small cell undifferentiated, mixed epithelial and mesenchymal with and without teratoid features (3).

The purely fetal epithelial type of HB is composed of cells arranged in one to two cell thick cords or trabeculae resembling normal fetal hepatocytes with a round centrally placed nuclei containing dispersed chromatin and single prominent nucleoli. On low

power examination, this subtype displays an alternate dark and light pattern resulting from the variable accumulation of glycogen and lipid within the cytoplasm of the tumour cells. Another characteristic feature of this subtype is the presence haematopoietic cells admixed within the tumour cell clusters or within the sinusoidal system, featuring extramedullary haematopoiesis like that seen in a normal fetal liver. The mitotic count is very low (<2/10hpf) when compared to the embryonal subtype and surgical resection is considered curative. This threshold of <2mitosis/10hpf was first described and defined in literature by Weinberg et al (27). It is also not uncommon to find tumours with all the features of fetal type as described above, but with increased mitotic activity of 2-10/hpf. This has been termed as 'mitotically active fetal HB' and is often found intermixed with the embryonal subtype (28).

In the embryonal subtype, the tumour cells are arranged in clusters and tubular/acinar structures around lumina, forming a pseudo-glandular pattern. The individual tumour cells are smaller than the normal hepatocytes with scant cytoplasm and a centrally placed round to slightly irregular nuclei containing clumped chromatin, high nuclear cytoplasmic ratio and displaying brisk mitotic activity. Often, this subtype is seen admixed with the fetal type and pure embryonal subtype is very rare (28).

The small cell undifferentiated subtype, as the name suggests is composed of undifferentiated/ blastemal cells with a diffuse growth pattern and lacking the trabecular, acinar or pseudoglandular pattern that classically define the fetal and embryonal subtypes. The cells forming this subtype are small with round nuclei displaying clumped chromatin, high nuclear cytoplasmic ratio and high mitotic activity. This subtype usually presents with normal or low levels of AFP and is found

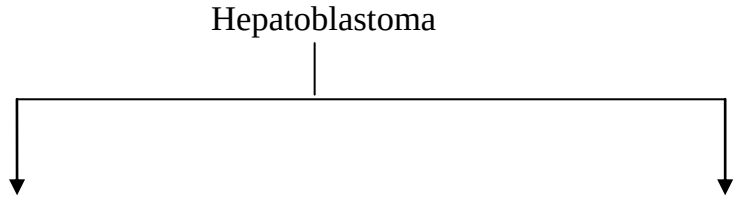
to have an aggressive outcome. Haas et al in a study has found a recurrence rate of 63% for this subtype and has also emphasized the importance of identification of the focal component of this subtype, when present, as they are associated with a higher degree of recurrence when compared to the other subtypes (5). However, these cells must comprise atleast 50% of the tumour area to be designated as SCUD subtype (29).

Macrotrabecular variant is a subtype of HB wherein the tumour cells are arranged in broad trabeculae of >10 cell thick plates (16). The exact criterion defining the number of plates is not uniform and is different in various studies. The first study on the histological patterns by Gonzalez et al defined this subtype to have >20 cell thick plates (30). The individual tumour cells that comprise this pattern may belong to the fetal, embryonal, large cell or the undifferentiated subtypes. They often resemble the adult Hepatocellular carcinoma, often called "HCC like" cells and are found to have an aggressive outcome. The term 'macrotrabecular' is restricted to those tumours with a predominant macrotrabecular pattern and not in cases where only a focus of this pattern is present (31).

A proportion of HB cases, in addition to the epithelial component (fetal or embryonal or both) display mesenchyme derived structures within the tumour. The mesenchymal components commonly present are mature and immature fibrous stroma, cartilage and osteoid like material. However, at rare instances, the tumour might contain other heterologous elements like mucin producing columnar epithelium, neuro-epithelium like structures, ganglia, bone, melanin pigment, skeletal muscle etc. This subtype with the heterologous elements is called mixed epithelial and mesenchymal(MEM) with teratoid features and the former, MEM without teratoid. However, the presence of

myofibroblasts, smooth muscle or squamous epithelium does not qualify to make a diagnosis of teratoid type (32). Thus this subtype also provides evidence that the tumour originates from pluripotent or hepatic progenitor cells with a capacity for differentiation along multiple lines.

Summary of the Armed Force Institute of Pathology (AFIP) classification(31):



Epithelial (56%)

1. Fetal (31%)
2. Fetal and Embryonal(19%)
3. Small cell undifferentiated (3%)
4. Macrotrabecular(3%)

Mixed epithelial and mesenchymal(MEM) (44%)

1. With teratoid features (20%)
2. Without teratoid features (80%)

Chemotherapy in HB:

The use of pre-operative chemotherapy has led to a remarkable decrease in the amount of viable tumour, however the number of cycles of chemotherapy has no effect on the extent of necrosis. This factor alone has been found to significantly associated with improved overall survival in a study conducted by Venkataramani et al (31). Necrosis of the tumour is often seen bordered by haemosiderophages and reactive and proliferating fibroblasts. Apart from necrosis, there are numerous histological changes encountered in HB following chemotherapy, of which osteoid formation has been reported to be the most prominent one, accounting for only <5% of the total surface area to a maximum of 40% in cases who received chemotherapy (34). Osteoid like

material, defined as acellular pale matrix surrounding an ovoid cell with hyperchromatic nuclei (osteoblast) is a common finding seen in cases classified as MEM subtype and adds to confusion while subtyping the tumour pre-chemotherapy, especially when seen as an isolated finding and when the clinical details are not provided. Other common findings seen in a tumour following administration of neo-adjuvant chemotherapy are hyalinisation, fibrosis, giant cell reaction, squamous differentiation, fatty change, calcification, haemorrhage, ductular and vascular proliferation (35). There is no preference for ablation of a particular tumour type following chemotherapy and the application of pre-operative chemotherapy helps in the maturation/ differentiation of the tumour(36).

Staging systems:

Accurate staging of the tumour is essential for precise staging and classification of the risk groups so that prognostic and predictive factors can be determined. Various systems have been proposed and followed since 1989 for staging of HB. The major study groups involved in this aspect are the International childhood Liver tumour strategy group (SIOPEL), Children's oncology group (COG), German Society for Pediatric Oncology (GPOH) and Japanese study group for Pediatric Liver Tumour (JPLT). These groups have proposed risk stratification of HB patients based on number of factors. The most widely accepted and used classification systems are SIOPEL (36) and COG (37).

SIOPEL System:

The SIOPEL group designed the PRETEXT (Pretreatment Extent of the disease) staging system for staging and risk stratification of HB which serves as a wonderful tool in the prediction of resectability and survival. According to this protocol, the respectability of the tumour is assessed before administration of chemotherapy by imaging modalities like ultrasonogram, CT, MRI etc. The original site and extent of the tumour is denoted by the sections of liver involved and the stage is represented as PRETEXT I - IV. The term 'section' is used to avoid confusion with sector and segments and is based on the Couinaud's system of division of liver into eight segments(36). The right lobe of liver is divided into right posterior (segment 6&7), right anterior (segment 5&8), left medial (segment 4a&4b) and left lateral (segment 2&3). The PRETEXT staging is outlined in brief below (36). Additionally, various parameters like the focality (F), caudate lobe involvement (C), extrahepatic spread (E), portal vein involvement (P), hepatic vein or vena caval involvement (V), tumour rupture (H), nodal spread (N) and distant metastasis (M) are assessed and denoted by the corresponding alphabet mentioned above as and when applicable. Then the tumour is subjected to biopsy and histopathological evaluation before starting chemotherapy. After a course of 2-4 cycles of neo-adjuvant therapy, the tumour is restaged and this has been called POST-TEXT (Post treatment extent of the disease). In a study by Venkataramani et al, downstaging of tumour as per the POST-TEXT system was found in a significant number of patients (38). Thus, this system gives knowledge about the morphology of the tumour prior to and after chemotherapy.

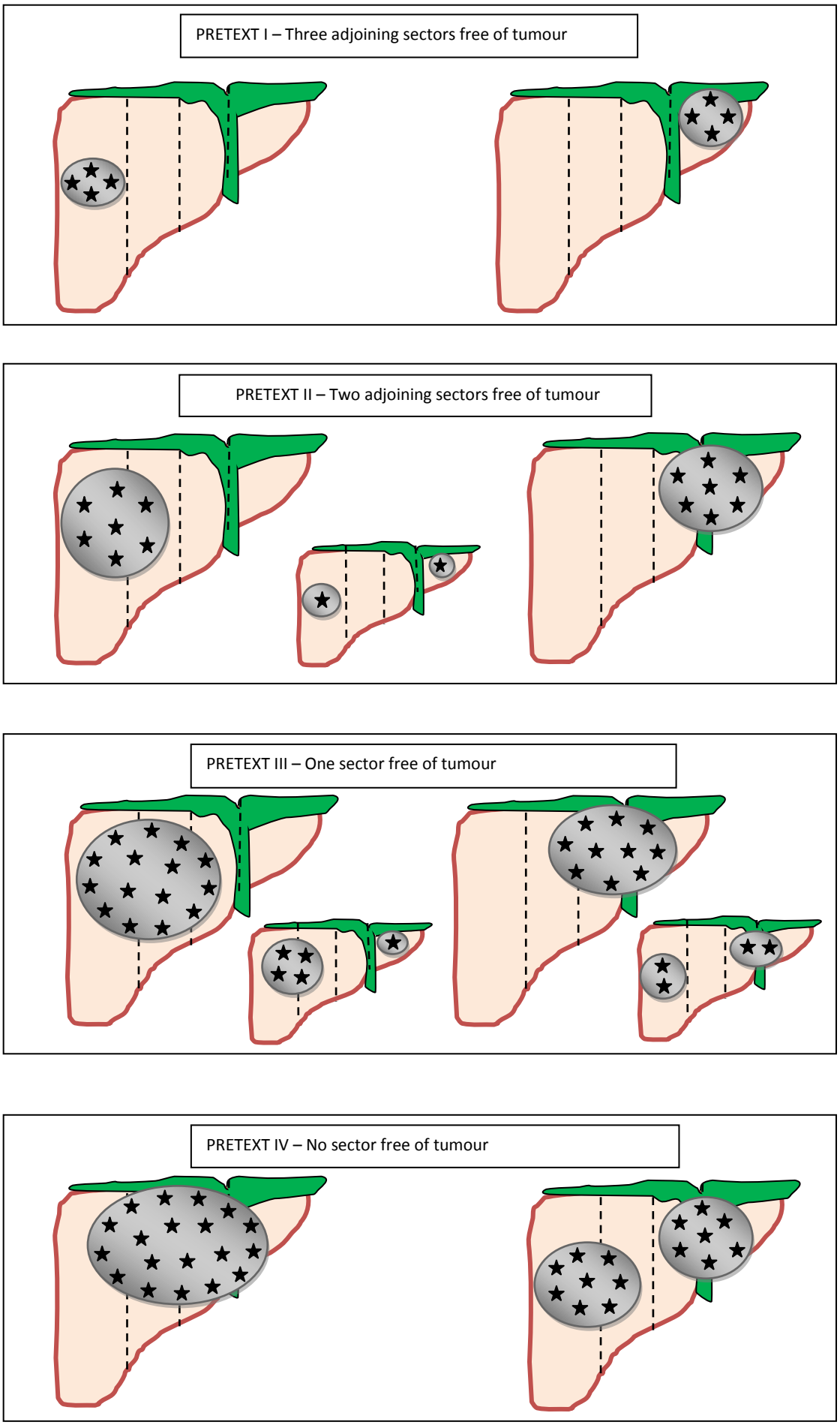
PRETEXT staging:

PRETEXT I: Involvement of one section, with three adjoining sections free of tumour.

PRETEXT II: Involvement of one/two sections, with two adjoining sections free of tumour.

PRETEXT III: Involvement of two/three sections and no two adjoining sections free of tumour.

PRETEXT IV: Involvement of all four sections.



PRETEXT classification of Hepatoblastoma - Adapted fromRoebuck et al from the SIOPEL group (36).

Furthermore, the patients are divided into standard risk (PRETEXT I-III, no extrahepatic disease/metastasis/vascular involvement) and high risk (PRETEXT IV, extrahepatic disease, AFP<100ng/mL and tumour rupture) (39). According to SIOPEL 1 study, the five yearoverall survival was 100% for patients under PRETEXT 1 and 57% for PRETEXT IV.

Evans (COG) staging system:

This was devised by the North American study group which stratifies HB patients into four groups based on the extent of surgical resection prior to administration of chemotherapy. Hence, in all tumours where surgical resection was feasible, resection was the primary treatment followed. The tumour was classified into one of the four groups, based on the per-operative and histopathological findings. The decision on the administration of chemotherapy was made later based on the stage and risk category of the tumour. The staging system proposed by the COG and the risk stratification are described below (37).

Stage I: Tumour completely resected at diagnosis with negative margins.

Stage II: Tumour completely resected at diagnosis with microscopic tumour involving the margins.

Stage III: Only biopsy at diagnosis or complete resection with involvement of nodes or spillage of tumour or incomplete tumour resection with residual intra-hepatic disease.

Stage IV: Metastatic disease at the time of diagnosis.

A risk stratification system was also described by the COG, according to which the tumours are stratified into four groups: very low risk to high risk as follows:

Very low risk: Pure fetal histology, Stage I/II

Low risk: Any other histology, Stage I/II

Intermediate risk: SCUD histology, Stage III

High risk: Stage IV, AFP<100ng/mL

Although both the staging systems have insisted that surgical resection is the primarymodality of treatment in HB, PRETEXT system of risk stratification is considered to be superior in predicting the survival rates when compared to COG system (40). This is because only in about one-third to one-half of the patients the tumour is resectable at diagnosis (41) and hence the COG system of classification cannot be applied. In order to overcome the differences in the stratification of patients and to provide a uniform risk stratification system, Children's Hepatic tumour International Collaboration (CHIC) was formed which consolidated the data from all the four groups SIOPEL, COG, GPOH and JPLT over a period of twenty years from 1989 to 2008. The data consisted of details of about 1605 patients and this will provide in depth knowledge about the various parameters that define the disease characteristics and outcome(36,40).

Molecular pathogenesis and the use of immunohistochemistry:

There are a number of molecular pathways implicated in the pathogenesis of HB which include the Wnt/Beta-catenin signalling pathway, Hepatocyte growth factor/c-met pathway, Notch and Hedgehog signalling pathway. Of these, the Wnt/Beta-catenin pathway has been of interest to many biologists in the recent years. Wnt/beta-catenin is a key regulator involved in intercellular adhesion, cell growth and differentiation. The activation of this pathway has been implicated in the proliferation of the stem cell compartment and thereby carcinogenesis in various tumours including the liver, breast, prostate and colon (42), with the highest percentage in HB (50-90%) (43). In a study conducted by Udatsu et al, around 81% of hepatoblastomas were found to be associated with mutations in beta-catenin and thereby activation of the Wnt signalling pathway(44). The key proteins involved in this pathway are the Wnt signals which bind to the Frizzled receptor on the cell surface, APC (Adenomatous Polyposis Coli) gene which is a tumour suppressor and helps in the degradation of beta-catenin, and beta-catenin itself. Beta-catenin is a multifunctional protein localized to the cytoplasmic aspect of a cell. In the resting state, when there are no signals through the Wnt pathway, the APC protein causes degradation of beta-catenin through phosphorylation of its NH2 terminal. In case of mutations in any of the proteins described above, beta-catenin accumulates within the cytoplasm. It then translocates to the nucleus and binds to nuclear transcription factors like T-cell factor (Tcf) and lymphoid enhancer factor (Lef). Thus progression of cell cycle is enhanced by further activation of target genes like e-cadherin, cyclin D1, c-myc etc(44–46). Mutations in the degradation targeting box of the gene that codes for beta-catenin

(CTNNB1 exon 3) is the most common and has been found in around 75% of HB (46). A simpler way of identification of the derangement in this pathway is the application of immunohistochemical method to identify the expression of beta-catenin antigen in the tumour cells and compare with the expression in normal liver tissue. In the latter, beta-catenin expression is limited to the cell membrane of hepatocytes, bile ducts and ductules. In the tumour cells, beta catenin is expressed in the nucleus with or without cytoplasmic expression.In a study conducted by W.S.Park et al, in the Korean population, about 30 cases of hepatoblastoma were analyzed with respect to immunohistochemical staining of beta-catenin, of which 29 cases showed positivity. They also found that nuclear localization of beta catenin is comparatively higher in the embryonal or undifferentiated subtypes. Furthermore, it was found that nuclear beta catenin staining correlated with advanced clinical stage, undifferentiated subtype and a shorter survival(6). Till date, there is no universal scoring system for the immunohistochemical expression of beta-catenin. Sarangarajan et al has devised a scoring system, according to which the expression is measured with respect to the pattern of staining – membranous, cytoplasmic and nuclear and graded the intensity from 0 to 3+ - no staining, mild, moderate and intense staining. In this study, it was also found that there is no difference in the intensity of staining in the post-chemotherapy specimens in comparison to the pre-chemotherapy biopsies in the fetal subtype, whereas a marginal decrease was observed in other subtypes(47). The importance of identification of beta-catenin mutations also relies in the fact that the subset of patients who relapse after conventional treatment can be put into clinical trials in which the drugs targeting the beta-catenin pathway can be targeted. However,

the role of immunohistochemistry alone in this regard is controversial and this has to be confirmed by identification of the mutation of the beta-catenin gene.

Hepatic progenitor cells in HB:

The varied range of epithelial and mesenchymal differentiation in HB implies that the tumour originates from cells with pluripotent properties. There are two types of pluripotent cells in human liver – the hepatoblasts and the hepatic stem cells. They are localised to the canals of Hering which connect the biliary canaliculi to the interlobular bile ducts (48). It has been found that these two different types of hepatic progenitor cells reside in different portions of Canals of Hering in the fetus and in the adult liver. It has been proved using immunohistochemical marker expression that the hepatic stem cells are the precursors to the hepatoblasts which are rapidly proliferating and possess the capacity to differentiate into hepatocytes as well as the cholangiocytes (49).

The immunohistochemical profile helps to differentiate the various subtypes of HB, according to the stage at which the malignant transformation and block in maturation has occurred. Studies have found that CK-19 is expressed in 100% of embryonal areas and about 66% in the fetal areas (50) and a higher disease stage correlated with CK19 expression(7). CD326 or EpCAM (Epithelial Cell Adhesion Molecule) is a homophilic calcium ion independent glycoprotein molecule, involved in cell-cell adhesion, expressed in many human epithelial tissues including gall bladder, liver, ovary and breast. EpCAM is vital in various processes of cell biology including adhesion, intracellular signaling mechanisms, cell migration and differentiation. The

results of the study conducted by de Boer et al suggested that the hepatic progenitor cells with a proliferative phenotype are associated with an expression of EpCAM (9). The expression of EpCAM is found to be present in early stages of oncogenesis of Hepatocellular carcinoma (HCC) and associated with poor prognostic subtypes (51,52) . The previous studies in the literature suggest that EpCAM is expressed in 70-80% HBs and it is 100% positive in the epithelial areas (52). Furthermore, in HCC pathogenesis, EpCAM was identified as a target gene in the Wnt/beta-catenin signaling pathway, with the tumours expressing EpCAM showing a response to the drugs targeted against beta-catenin (53). In addition, recently EpCAM specific monoclonal antibodies were developed and found to be of great value in tumours expressing this marker, irrespective of the previous treatment with cisplatin based chemotherapy (10).In the previous studies, >5% of tumour cells expressing the markers were considered positive(7,54).

Embryology and immunohistochemistry:

Haematopoiesis begins in the fetal liver by around 6weeks of gestation, with a peak at 12weeks and it stops as the bone marrow takes over this function at around 35-36weeks. The production of erythrocytes is largely confined to the parenchyma of the liver, while the granulopoiesis and megakaryocyte production occurs within the portal tracts (16). In many cases, it becomes difficult to differentiate normal infantile liver tissue with features of extramedullary haematopoiesis from that of neoplastic tissue composed entirely of fetal epithelial component. In this scenario, the alpha-fetoprotein levels may not help in the diagnosis because of the normally elevated levels of AFP in

the first 6months of life. On H&E examination, normal liver tissue consists of lobules of hepatocytes with central veins and portal tracts with the hepatocytes arranged in cords of 1-2 cell plate thickness with uniform polygonal cells featuring a central round nucleus and abundant pale eosinophilic granular cytoplasm. The pure fetal epithelial type of HB also shows similar cytological features, while lacking the portal tracts and the central vein. However, in a biopsy sample, it becomes a diagnostic difficulty, especially for a neophyte in liver pathology. Immunohistochemistry using the previously mentioned markers might be of value in this setting. Hep Par 1 stains both the normal and neoplastic liver tissue and cannot be used in differentiating both. Similarly, both the normal liver as well as the fetal type HB show a membranous pattern of staining for beta-catenin and does not help in the diagnosis. However, EpCAM does not stain the normal liver tissue, while the neoplastic cells express a strong and diffuse membranous pattern of staining thus helping in making the correct diagnosis(10).

Management of Hepatoblastoma:

The overall five year survival rate of children with HB has shown a remarkable increase from only 35% in 1970s to as high as 75% in the 21st century (55). This can be attributed to the improvement in the management protocols followed in HB starting with surgical resection of the tumour to the current practice of resection following neo-adjuvant chemotherapy. The success of this modality of treatment has also been evidenced by other studies in addition to SIOPEL-1 (38,55,56). In a study by Medary et al, the tumour volume reduction ranged from 67% to 98% in three HB patients (57).

Chemotherapy not only causes shrinkage of the tumour, but makes the tumour less prone to bleed, leads to better delineation of the tumour from the surrounding normal liver parenchyma and helps to control the residual foci of microscopic tumour. Various drugs in combination have been used for HB including cisplatin, doxorubicin, vincristine, adriamycin, cyclophosphamide and 5-fluorouracil, of which the first two have proved to be most effective. PLADO combination (cisplatin – PLA and doxorubicin - DO) or monotherapy employing cisplatin, vincristine etc is the current modality of treatment for HB and is individualised according to the PRETEXT, POST-TEXT and the risk category of every individual(11,58).

In addition to chemotherapy, a number of recent developments in the treatment protocols have emerged that include hepatic artery chemoembolisation (HACE) and orthotopic liver transplantation (OLT), of which the latter has gained importance (11). OLT is employed as a primary modality of treatment for those patients with multifocal HB, involving all the segments of liver, HB with portal vein/hepatic vein invasion and intrahepatic residual disease, relapsed HB. This can also be used for tumours that do not show a significant clear margin by radiography. Primary OLT has shown significantly high rate of overall survival of 82% when compared to only 30% in cases in whom transplantation was done after failure of initial treatment (58).

Prognosis:

There is a drastic improvement in the survival of patients with HB over the past 20 years. This is attributed to the standardization of chemotherapy and resection of the tumour. In India, the survival rates of children with HB range from 33-100% and the main causes leading to failure of treatment are progression of the disease and the toxicity of the chemotherapeutic agents used (59). The overall prognosis of HB depends on a number of factors including the clinical presentation, AFP levels, extent of tumour, histological subtype and the presence or absence of metastasis. Of these, the gross resectability of the tumour has been found to be the most important factor predicting the prognosis(60). AFP level in the serum, not only stands as a useful tool in the diagnosis of HB, but also is of tremendous value in predicting the survival and prognosis. Low AFP levels at the time of diagnosis (<100ng/ml) and failure of AFP levels to return to normal range following chemotherapy have been proved to be of adverse prognostic value. Rise in AFP levels in a patient who is declared to be completely cured serves as an early indicator of relapse(61,62). As previously described, for the pure fetal epithelial type HB, primary resection is considered to be of complete cure and post-operative chemotherapy is not needed (4). The small cell undifferentiated subtype has also shown to be of poor prognostic outcome, even when present focally and thus warrants a thorough search for this pattern (5). In a study by Davies et al, it was found that viable tumour following chemotherapy correlated with cure rates and survival. Other factors like multi-focality of the disease and the invasion of small vessels also predict tumour related morbidity and mortality(63). The presence of heterologous elements in HB following chemotherapy has been attributed

to the maturational effects and selection of mature clones by chemotherapy. These elements have been found to be associated with a good prognosis in studies by Haas et al and Stocker et al(31,62). However, Kasai et al found these changes to be of no prognostic significance(64). About 20% of HB patients present with distant metastasis at the time of diagnosis, the most common site being the lung (65). In a series by Wanaguru et al, children with pulmonary metastasis had a higher rate of relapse and a poor outcome when compared to children without metastatic disease (66). Excision of the metastatic lung tumours have also been advocated by some authors and have been proved to be of benefit (67).

MATERIAL AND METHODS

MATERIAL AND METHODS

The material for this study comprised of all cases of Hepatoblastoma (HB) diagnosed in the Department of General Pathology, Christian Medical College and Hospital, Vellore, India during the period from January 2000 to March 2015. The biopsies as well as the resection cases were included in the study. The pathology report details were obtained from the department online data base. A total of 55 patients were diagnosed to have HB during this study period. Of these, 22 patients had only biopsies, 11 had resections alone and in 22 patients both biopsy and resection materials were available. Tumour recurrence was noted in three patients, of which two had metastatic tumour as well. In addition, another patient had resection of metastatic tumour.

Slides and blocks were retrieved from the archives of Department of Pathology. All specimens were fixed in 10% formalin and were embedded in paraffin.Four micron thick sections were cut and routinely stained with haematoxylin and eosin. Periodic acid Schiff with and without diastase, and Foot' reticulin stains were done when necessary.The slides were reviewed and a detailed study on the histopathological features was done with recording of the data. Representative blocks of the tumour were selected for immunohistochemical studies.

Clinical laboratory and radiological parameters:

The relevant clinical details were abstracted from the online data base stored in the Department of Pathology, which included the following: Age, sex, presenting complaints such as abdominal distension, abdominal mass, failure to thrive, jaundice,

fever and loss of weight and appetite. Incidental detection of the tumour and the duration of the symptoms were noted. History of previous chemotherapy, the durationand the number of cycles were also noted.

The laboratory parameters taken for evaluation in this study included serum levels of alpha fetoprotein (AFP) and beta-hCG (human chorionic gonadotropin). Serial measurements of AFP were also noted at the time of diagnosis, post-chemotherapy, post-operative and at the time of last follow up.

Radiological findings of the ultrasonogram (USG) and computed tomography (CT) (wherever available) were noted with special emphasis on the size and location of the tumour, focality, involvement of major veins and presence of metastasis. Wherever available, the PRETEXT (Pretreatment extent of disease) staging was also noted.

Pathological Evaluation:

Macroscopic features:

Biopsy specimens: All biopsy specimens were evaluated in terms of nature of the biopsy – trucut, USG guided and CT guided. The number of cores and the length of each core of tissue were noted. The aggregate total and mean lengths were then calculated.

Resection specimens: The tumour location, number, size, appearance (solid/cystic) and the presence or absence of necrosis within the tumour were recorded.

Microscopic features:

The number of histological sections available for each resection case was recorded.

Microscopic examination of the slides was done in detail and the histological parameters analyzed in both the biopsy and the resection specimens included the pattern of arrangement of the tumour cells, histological subtype, mitotic activity/10 high power fields (hpf), presence or absence of extramedullary haematopoiesis, cholestasis and fatty change within the tumour.

Histologically, the tumours were classified into six major histological groups : pure fetal epithelial, mixed embryonal and fetal epithelial, macrotrabecular, small cell undifferentiated, mixed epithelial and mesenchymal with and without teratoid features (3). Pure fetal epithelial tumours exhibited a thin trabeculae of cells which resembled the fetal hepatocytes and had a small round central nucleus and clear to granular cytoplasm, giving an appearance of 'light and dark' pattern on low power examination. Pure fetal type tumours had mitosis <2/10hpf and clusters of haematopoietic cells, resembling the extramedullary haematopoiesis seen in normal fetal liver. A tumour was classified as 'mitotically active fetal' type when the mitosis was >2/10hpf and this is often seen intermingled with the embryonal or the mitotically inactive fetal type (28). An embryonal subtype was designated when the tumour had a glandular, acinar or pseudo-rosette arrangement of cells, with the individual cells being small and angulated with a hyperchromatic nucleus and increased mitotic activity. Small cell undifferentiated pattern consisted of sheets of non-cohesive small cells with high nuclear: cytoplasmic ratio and a brisk mitotic activity. A

macrotrabecular subtype was diagnosed when the tumour cells were arranged in trabeculae of >10 cell thickness.

A tumour was classified under mixed epithelial and mesenchymal when the tumour exhibited mesenchymal derivatives like osteoid, bone, cartilage and fibrous stroma in addition to the epithelial component. When other heterologous elements like columnar epithelium, squamous epithelium, melanin, skeletal or smooth muscle, neural tissue etc were seen, the tumour was classified as mixed epithelial and mesenchymal – teratoid type(16,28,68).

A tumour was assigned a category in this study, depending on the predominant epithelial subtype (≥ 60%) exhibited. Those tumours with 60:40 ratios of two components or more than two components were classified as mixed subtype. However, for final diagnosis and statistical analysis, the tumours were classified as predominantly fetal epithelial (includes pure fetal and predominantly fetal), mixed epithelial type (includes predominantly embryonal and mixed fetal and embryonal), small cell undifferentiated (SCUD) and mixed epithelial and mesenchymal with and without teratoid (MEM).

Mitotic activity was counted for 10 high power fields and a cut off value of 5/10 hpf was taken to designate tumours into low or high mitotic rate categories. Cholestasis was identified by the presence of granular yellow brown pigment within the hepatocytes (hepatocellular type), brown bile pigment within the intercellular canaliculi (canalicular type) or within the periportal bile ductular structures (ductular type)(69). Steatosis was identified by the presence of fat droplets in the cytoplasm of

the tumour cells and was qualified as microvesicular, macrovesicular or mixed type. The degree of steatosis was graded as mild: <30%, moderate: 30-60% and severe: >60%(70).

In the post-chemotherapy specimens, various effects of chemotherapy such as hyalinisation (graded as focal/extensive), presence of necrosis (focal/ extensive), foreign body giant cell reaction, keratin pearl formation/ squamous differentiation, recent and old haemorrhage (haemosiderophages) and presence or absence of calcification were also analyzed. All the post chemotherapy cases were examined carefully and the approximate percentage of viable tumour was documented to assess the response to chemotherapy. The percentage of viable tumours were graded as ≤25%, >25-<50% and ≥50%.

In all the tumours, the presence of microvascular invasion was documented. The distance of the tumour from the surgical resection margin was measured microscopically and was categorized as ≤0.5cm, 0.6 - <1cm and ≥1cm.

Recurrence and metastasis:

Similar macroscopic and microscopic features were assessed in all the resected recurrent and the metastatic tumours.

Immunohistochemistry:

The paraffin blocks of all biopsy cases which were available with adequate tissue were included for immunohistochemical studies. For all resection cases, the most appropriate and representative block from each case was chosen for

immunohistochemical (IHC) studies. For all tumours with mixed fetal and embryonal components, the block containing both the components was chosen. If there is no viable tumour in the post chemotherapy sample, IHC was not carried out. IHC of the biopsy sample and the corresponding post chemotherapy resection (wherever available) was done on the same slide for easy comparison of the pre and post chemotherapy changes. IHC was also carried out on the recurrent and metastatic tumours and the results were compared with the initial tumour. Cases with inadequate tissue material or no paraffin blocks available were evaluated with regard to the morphological subtype only and immunohistochemistry was not carried out.

Three immunohistochemical markers were used in this study – CK-19 (Cytokeratin-19), Beta-catenin and EpCAM (Epithelial cell adhesion molecule).

The details of the immunohistochemical markers used in this study are outlined below. All the antibodies were purchased from Leica and IHC was carried out using Ventana Benchmark XT. The detailed protocol for immunostaining is given in Appendix I.

ANTIBODY	DILUTION	CLONE	POSITIVE EXTERNAL CONTROL
CK-19 (Std 40)	1:150	b170	Ureter
EpCAM (Std 32)	Pre-diluted (RTU)	VU-1D9	Small intestine
Beta-Catenin(Std 40)	1:100	17C2	Small intestine

Evaluation of the tumour for immunohistochemistry:

For CK-19, all tumours were evaluated as positive or negative. The intensity of staining was graded as mild, moderate and strong. Positivity was defined as >5% of tumour cells expressing moderate or strong membranous staining for the marker. The percentage of positively staining cells was noted and classified as 5-25%, 26-50% and >50%. The expression of CK-19 is membranous in the biliary ductular epithelium, which served as an internal control and a known external control (ureter) was run with every batch of staining. Staining for CK19 was assessed in the fetal and embryonal subtypes separately.

Expression of beta-catenin was categorized as no staining/membranous staining, cytoplasmic±membranous staining (M+C), only nuclear staining (N) and nuclear+cytoplasmic staining (N+C). Those tumours with N and N+C were taken as positive and the remaining were taken as negative for statistical analysis. The percentage of tumour cells showing nuclear positivity for the marker was also noted and was categorised as 1-<5%, 5- 25%, 26-50% and >50%.The intensity of staining was assessed and reported as weak, moderate and strong. Any tumour with nuclear (N/ N+C) expression of beta-catenin in >5% cells with moderate/strong staining was taken as positive for the statistical analysis (35). The normal hepatocytes,the bile ducts and the ductules which exhibited a membranous pattern of staining were taken as positive internal control. In addition to this, with every batch of staining a known external positive control (small intestine) was run.

Antigen expression for EpCAM was defined as positive when the tumour cells showed a membranous pattern of staining. Expression of EpCAM was evaluated using the Spizzo's scoring system(71), according to which a total immunostaining score (TIS) was calculated as the product of proportion score (PS) and intensity score (IS).

Proportion score (PS) was assessed based on the percentage of positively staining tumour cells (membrane expression).

No staining: 0; 1- <10%: 1+; 10-50%: 2+; 51-80%: 3+;>80%: 4+

Intensity score (IS) was based on the intensity of staining of the tumour cells.

No staining: 0; Weak staining: 1+; Moderate staining: 2+; Strong staining: 3+

Thus, TIS was calculated as,

TIS = PS × IS and ranged from 0 to 12, with nine possible values (0, 1, 2, 3, 4, 6, 8, 9 & 12).

The final EpCAM expression was graded as,

Weak: TIS 0 to 4, Moderate: TIS 6 & 8, Intense: TIS 9 & 12.

The bile ducts and the ductules which exhibited a membranous pattern of staining were taken as positive internal control. In addition to this, with every batch of staining a known external positive control (small intestine) was run.

Statistical methods

The data was analyzed using excel spread sheet and statistical analysis was done using SPSS software Version Stata/IC 13. Frequency along with percentages was used to denote categorical variables and mean along with standard deviation for continuous variables. Chi-square/ Fisher's exact test was used to compare the association between categorical variables and a 'p' value of less than 0.05 was considered significant. The overall survival (OS) and event free survival (EFS) were calculated. Event was described as death/metastasis/ recurrence. Kaplan-Meier curve was used to depict the survival and log rank test was used to compare the survival in different groups.

RESULTS

RESULTS

Slides and paraffin embedded tissue blocks from a total of 55 cases of HB were obtained from the Department of Pathology, Christian Medical College and Hospital, Vellore, from January 2000 to March 2015. This included 22 biopsies, 11 resections and 22 cases with both biopsy and resection. Thus, a total of 44 pre-chemotherapy biopsies and 33 post-chemotherapy resections were included in the study. Three patients who were treated surgically for the primary tumour underwent resections for recurrent tumour after 88 months, 29 months and 16months respectively and two of these patients also underwent resection of the metastatic tumour, 132 months and 14 months after the initial resection, respectively. One other patient also underwent resection of the metastatic tumour in the lung, 7 months after the initial diagnosis.

Demographic profile:

The mean age of the patients at presentation was 2.33±2.47 years with an overall range of 0.05 – 13 years (n=55) (Fig 1). The youngest patient was a male child who was diagnosed to have a liver mass inutero and was operated at 20 days (0.05 years) of life. The male: female ratio was 2.1:1 (37 males and 18 females).

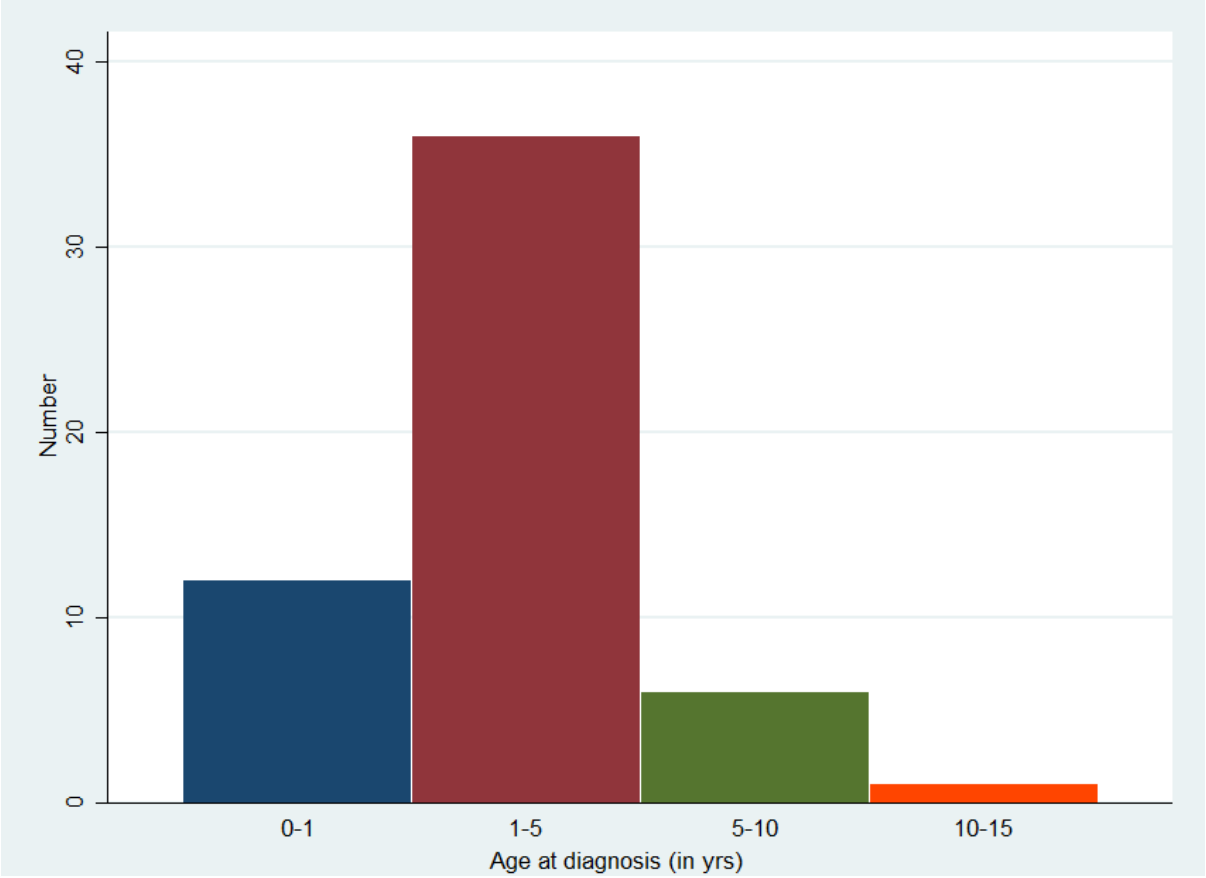


Figure 1: Age distribution in HB patients (n=55)

Clinical profile:

The details of the clinical presentation were available for 52 patients and the most common clinical presentation was abdominal mass (38.18%) followed by fever (31%) and abdominal pain (14.6%). Two patients presented with irritability and upper respiratory tract infection and during evaluation an abdominal mass was incidentally detected. One patient, a 20 day old male infant was diagnosed to have a liver mass in utero, during routine antenatal ultrasonography (Table 1).

Table 1: Clinical presentation of patients with HB(n=52)

Presenting symptoms	Number (n=52)
Abdominal mass	21 (38.2%)
Fever	17 (31%)
Abdominal pain	8 (14.6%)
Loss of weight/appetite	7 (12.73%)
Jaundice	4 (7.27%)
Incidental	4 (7.27%)
Failure to thrive	3 (5.45%)
Antenatal	1 (1.82%)

Associated findings:

Two patients (3.63%) with HB were also found to have neuroblastoma. The first patient was a four month old male child who was first diagnosed to have neuroblastoma of the left adrenal gland. Later at two years of life, he presented with abdominal distension and was found to have elevated levels of AFP and a large heterogenously enhancing mass in the right lobe of the liver, which was diagnosed as HB, pure fetal epithelial subtype. The second patient was a two year old male child who presented with abdominal distension and fever. He was found to have elevated serum AFP level and a well defined heterogenous mass in the right lobe of liver, which was diagnosed as HB, pure epithelial (mixed fetal and embryonal) subtype.

Three months later, he was found to have a large heterogenous mass in the posterior mediastinum extending from D3-D10, which was diagnosed as neuroblastoma.

The other associations found in our patients were the presence of horseshoe kidney in two patients, of which one also had medullary sponge kidney and gonadal enlargement with precocious puberty and gastroschisis in one each.

Serum alpha-feto protein:

Serum AFP level at the time of initial diagnosis was available for 46 patients, and ranged from 4.32IU/ml to 549400 IU/ml with a median of 30000IU/ml. At diagnosis, only one patient (2.17%) had normal serum AFP level (4.32IU/ml) and was diagnosed to have SCUD type of HB. A decrease in the serum AFP levels following chemotherapy and following surgery was observed in 22/28 (78.57%), while six patients (22.43%) had persistent increase. The values of AFP at diagnosis, post-chemotherapy, post-operative and at the time of last follow-up are shown in Table 2. It was observed that there is a significant difference in the decrease in AFP levels when the values at initial diagnosis and post-chemotherapy were compared (p=<0.001*). Similar results were seen when the post-chemotherapy levels were compared with the post-operative levels (p=<0.001**) (Fig 2)

Table 2: AFP levels measured at various periods in HB patients

Serum AFP (No)	Median (IU/ml)	Range (IU/ml)	P value
At diagnosis (28)	300000	4.32-549400	<0.001*
Post-chemo (27)	2390	2.68-300000	<0.001**
Post-op (22)	42.1	1.13-11800	0.14
At last follow up (19)	2.31	0.2-300000	

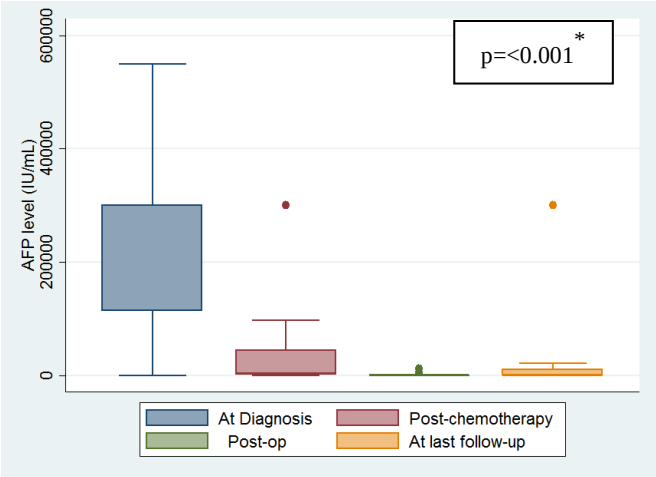


Figure 2: Box plot depicting the AFP levels in HB patients measured at various period of time.

Serum beta-hCG:

Serum beta-hCG was found to be elevated in 3/55 (5.45%) of patients, of which one was a male child who presented with precocious puberty (167mIU/ml) and the other two were female children with 53.8mIU/ml and 17.11mIU/ml. Two of the three children (66.67%) with high values of beta-hCG died of disease.

PRETEXT classification:

The detail about the PRETEXT of the tumour was available in 25 patients. Majority of patients 13/25 (52%) belonged to PRETEXT-II and 6/25 (24%) belonged to PRETEXT-III. There were 3 patients in PRETEXT-I (12%) and PRETEXT-IV (12%)(Fig 3).

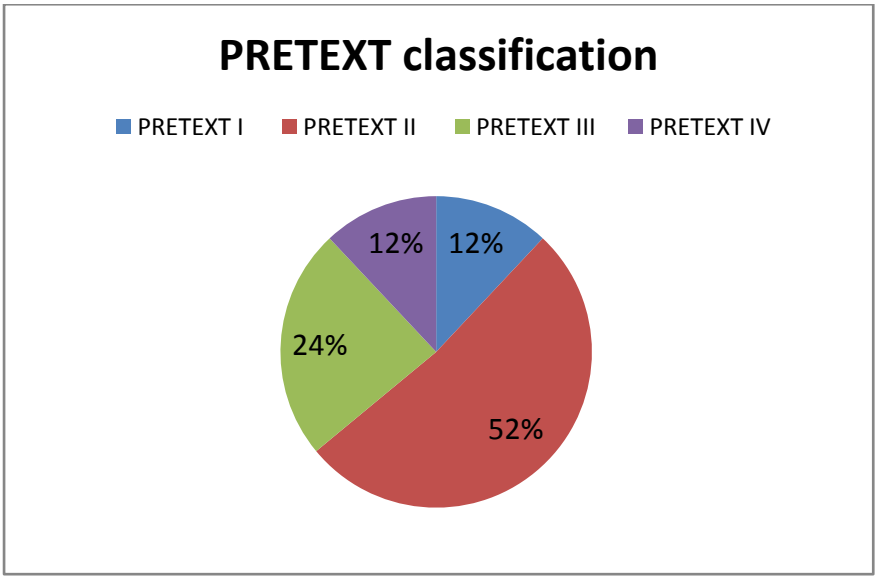


Figure 3: PRETEXT in HB patients(n=25)

Details about the chemotherapy:

Of the 33 patients in whom a resection was done following chemotherapy, the details about the number of cycles of chemotherapy were available in 27 patients (27/33). The average (range) number of cycles of chemotherapy given was 4.73±1.08 (3-8).

Nature of the biopsy:

Of the 44 patients who had a biopsy prior to chemotherapy, the detail about the type of the biopsy was available in 40 patients. A majority of the patients 26/40 (65%)

underwent tru-cut biopsy of the lesion, followed by USG guided in 12/40 (30%) and CT guided in 2/40 (5%).

Tumour characteristics – Radiological, macroscopic and microscopic features:

Radiology: Tumour location:

Of the 55 tumours, 30 (54.55%) were present in the right lobe, 11 (20%) in the left lobe and 14 (25.45%) involved both the lobes (Fig 4).

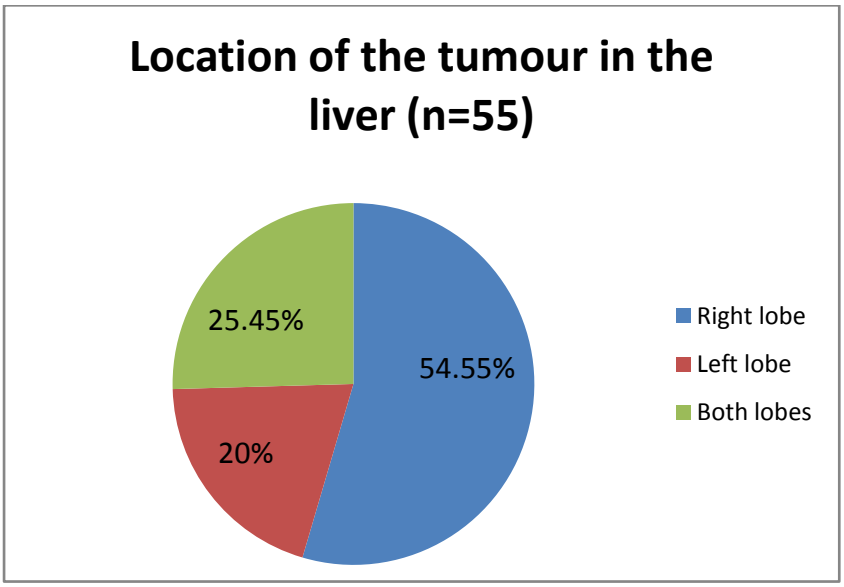


Figure 4: Location of the tumour in the liver by radiology(n=55)

Tumour focality:

The details about the focality of the tumour, whether single or multiple were available for 37 patients, of which 30/37 (81.08%) had a single tumour nodule while 7/37 (18.91%) had multiple tumour nodules in the liver.

Tumour dimensions:

The detail about the size of the tumour at the time of initial presentation by radiology was available for 43 patients and the average (range) size was 10.06±3.12 (3.5-18) cm. Following chemotherapy, the gross dimension was measured in 32/33 tumours and the average (range) size was 6.70±2.89 (1.5-15) cm. Both the pre-chemotherapy (by radiology) and the post-chemotherapy (gross) measurements were available for 25/33 cases and the difference in the size of the tumour between the two groups was statistically significant (p=<0.001*) (Fig 5).

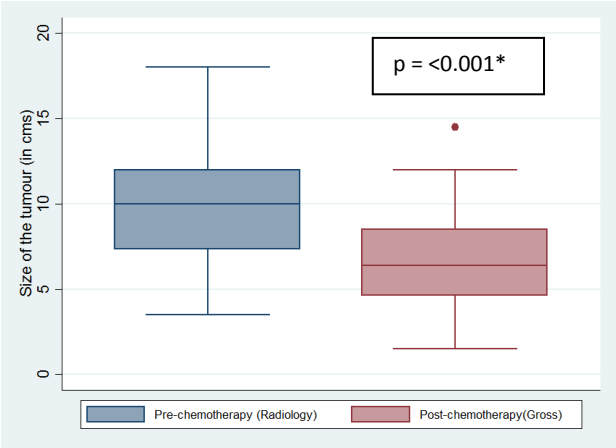


Figure 5: Box plot showing the size of the tumour in the pre and post chemotherapy groups.

Gross features:

Biopsy details:

Of the 44 biopsy samples in the pre-chemotherapy group, the details about the number of cores of tissue were available in 41 samples. In the remaining 3 cases, the paraffin blocks were received from elsewhere. The average number of cores was 6±3. The mean aggregate length of the cores was 1.93±1.31cm.The largest core was 2cm and the smallest was 0.1cm in length.

Details of the resected tumours:

Of the 33 resection specimens, 28 (84.15%) had a solid tumour and 5 (15.15%) had both solid and cystic areas. Necrosis was grossly evident in 8 (24.24%) cases.

Grossly, the tumours had a lobulated, firm, tan to grey white cut surface with haemorrhage, cystic change, fibrosis, necrosis, calcification and myxoid change in varying proportions.

For the resection specimens, the average (range)number of sections available for this study was 7.45±2.75 (4-16).

Histological Features:

Pattern of arrangement of the tumour:

The most common pattern of arrangement was trabeculae in 43/55 (78.18%) of tumours followed by cords and solid nests in 23/55 (41.81%). The other patterns were rosettes and primitive tubules, predominantly seen in the embryonal subtype. The different histologic patterns observed in HB are shown in Fig 6.

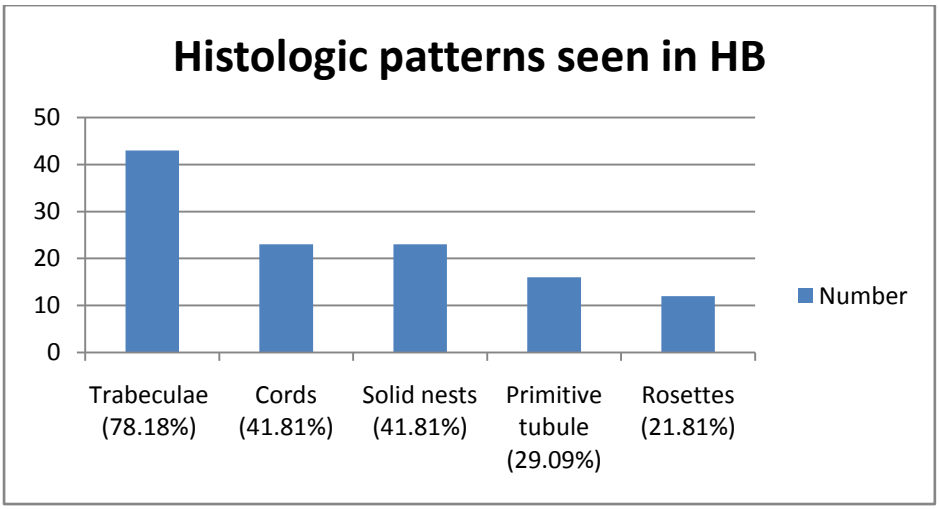
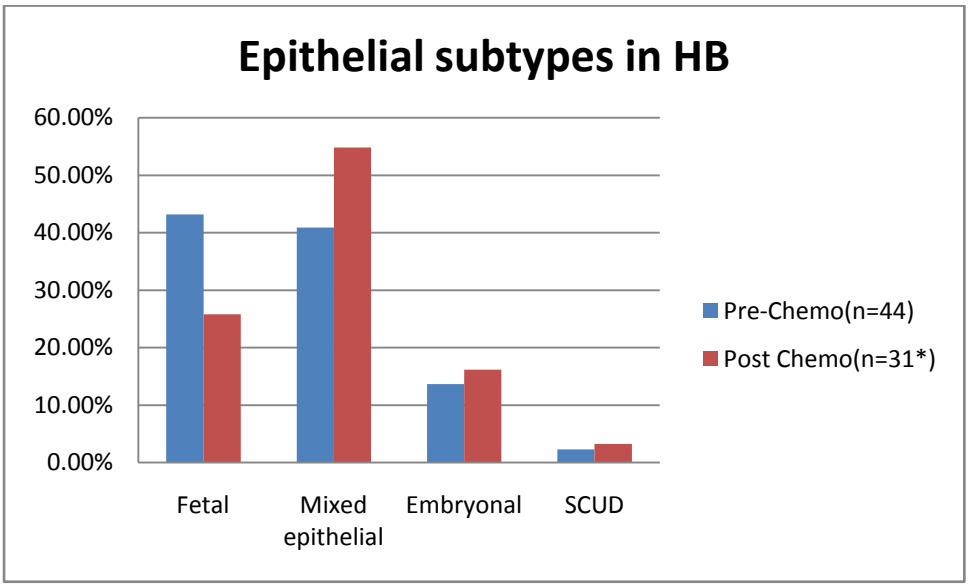


Figure 6: Different histological patterns seen in HB(n=55)

Epithelial subtypes:

The most common epithelial subtype in the pre-chemotherapy group was predominantly fetal subtype in 19/44 (43.18%) cases, which included five tumours (26.32%) of pure fetal subtype.Six out of 44 tumours (13.64%) had predominantly embryonal subtype. In the post-chemotherapy group, mixed epithelial subtype was the commonest 17/31(54.83%), which included five tumours (16.15%) with predominantly embryonal subtype. This was followed by fetal subtype in 8/31 (25.8%) patients. SCUD subtype was seen in one patient (Fig 7). In the post-chemotherapy group, two cases had focal SCUD areas in addition to the fetal and embryonal components and two cases showed no residual viable tumour.One recurrent HB had focal areas of macrotrabecular pattern, but pure macrotrabecular subtype was not encountered in this study.



* Of the total 33 patients in the post-chemotherapy group, two patients had complete response to chemotherapy with no viable residual tumour.

Figure 7: Epithelial subtypes in HB: Pre and Post-chemotherapy groups

Extramedullary haematopoiesis:

Extramedullary haematopoiesis (EMH) was noted in the fetal component in 21/44 (47.72%) tumours in the pre-chemotherapy group and 24/33 (72.23%) tumours in the post-chemotherapy group (Fig 8). Among the cells that constituted EMH, erythroids were the most common cell type. Megakaryocytes were present in 3/44 (6.82%) and 6/33 (18.18%) of tumours in the pre and post chemotherapy groups respectively.

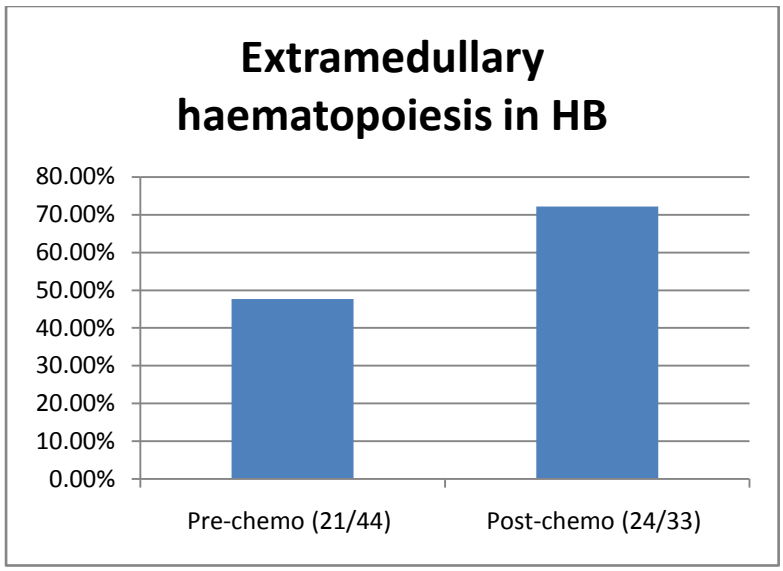


Figure 8: Extramedullary haematopoiesis in HB – Pre and post chemotherapy groups

Mitosis:

In the pre-chemotherapy group, the average number of mitosis was 2/10hpf in the fetal subtype, whereas it was 6/10hpf in the embryonal subtype. One patient in this group had a fetal type of tumour with mitosis of 15/10hpf and was categorized as 'mitotically active fetal type'. In the post chemotherapy group, the average number of mitotic figures was 1/10hpf and 11/10hpf in the fetal and embryonal subtypes respectively.

In the pre-chemotherapy group, 85.29% (29/34) of tumours with a fetal pattern and 36% (9/25) with embryonal pattern had a mitotic count of ≤5/10hpf. In the post chemotherapy group, 100% (18/18) of fetal and 47.05% (8/17) of embryonal subtype had a mitotic count of ≤5/10hpf and this difference was statistically significant (Table 3).

Table 3: Mitotic count in fetal and embryonal subtypes in the pre and post-chemotherapy groups

		≤5/10hpf	**>5/10hpf**	**p value**
Pre-chemo	Fetal (n=34)	29 (85.29%)	5 (14.71%)	
	Embryonal (n=25)	16 (64%)	9 (36%)	0.06
Post-chemo	Fetal (n=18)	18 (100%)	0 (0%)	
	Embryonal (n=17)	8 (47.05%)	9 (52.95%)	<0.001*

Cholestasis and steatosis:

Canalicular cholestasis was noted in 4/44 (9.09%) and 3/33 (9.09%) tumours in the pre and post-chemotherapy groups, respectively.

Steatosis was seen in 14/44 (31.82%) and 7/33 (21.21%) tumours in the pre and post-chemotherapy groups respectively. Microvesicular steatosis was common in pre-chemotherapy group (57.14%) whereas an equal number of tumours showed microvesicular and macrovesicular steatosis (42.85%) in the post-chemotherapy group. When the severity of steatosis was assessed, most of the patients in both the groups had only mild to moderate degree of steatosis and only 1/14 (7.15%) and

1/7 (14.28%) had severe degree of steatosis in the pre and post chemotherapy groups respectively (Table 4).

When steatosis was compared with the epithelial subtype, it was found that 100% of tumours with steatosis belonged to the predominantly fetal subtype and this was statistically significant when compared with embryonal and SCUD subtypes (p=0.03*) (Table 5).

Table 4: Steatosis – Type and severity in pre and post-chemotherapy groups in HB

		Pre-chemotherapy (n=14)	**Post-chemotherapy (n=7)**
Type	Microvesicular	8 (57.14%)	3 (42.85%)
	Macrovesicular	4 (28.58%)	3 (42.85%)
	Mixed	2 (14.28%)	1 (14.30%)
Severity	Mild	8 (57.14%)	3 (42.85%)
	Moderate	5 (35.71%)	3 (42.85%)
	Severe	1 (7.14%)	1 (14.30%)

Table 5: Comparison of predominantly fetal subtype with other subtypes - steatosis

	Predominantly fetal	**Others**	**P value**
Steatosis present	8 (100%)	0 (0%)	
Steatosis absent	11 (47.83%)	12 (52.17%)	0.03*

Mesenchymal elements:

Osteoid was noted in 8/44 (18.18%) and 25/33 (75.76%) and fibrous stroma in 2/44 (4.54%) and 12/33 (36.36%) tumours in the pre and post chemotherapy groups, respectively. One case in the pre-chemotherapy group had focal squamous differentiation in the form of keratin pearl formation.

Microvascular invasion:

Microvascular invasion (MVI) was present in 14/33 (42%) tumours in the post-chemotherapy group. Patients with MVI had lower EFS of 46.23 months when compared to those without (72.83 months), however this was not statistically significant (p=0.08).

Various subtypes of HB in the pre and post-chemotherapy groups:

The diagnoses in pre and post chemotherapy groups are given in Table 6. Two tumours in the MEM subtype had teratoid features. One patient was found to have mixed epithelial subtype of tumour in the pre-chemotherapy biopsy and melanin pigmentation was seen in the post-chemotherapy resection. Another patient had MEM subtype of tumour in the post-chemotherapy resection and developed metastasis in the lung. The metastatic tumour had neuroepithelium like structures (S100 and synaptophysin positive) and was classified as MEM with teratoid. Two patients had extensive ossification with complete response to chemotherapy and no viable tumour in the post-chemotherapy group.

Table 6: Various subtypes of HB in the pre and post chemotherapy groups:

Subtype of HB	Pre-chemotherapy (n=44)	Post-chemotherapy (n=31)
Predominantly fetal	17 (38.64%)	8 (25.81%)
Mixed epithelial	21 (47.73%)	11 (35.48%)
MEM	5 (11.36%)	11 (35.48%)
SCUD	1 (2.27%)	1 (3.23%)

Chemotherapy induced changes:

The various chemotherapy induced changes that were present in the post chemotherapy resection specimens include hyalinization, haemorrhage, haemosiderophages, necrosis, squamous differentiation, giant cell reaction, calcification and ossification (Fig 9).

Of the 55 tumours, six tumours (10.91%) had mature hepatocytes like pattern admixed with the fetal and embryonal subtypes. Multinodularity was seen in 4/55 (7.27%) tumours in which the tumour islands were separated by fibro-collagenous septa.

Other changes seen in the tumour were dense lymphoid aggregates in 3/55 (5.45%) tumours, nuclear vacuolization in 2/55 (3.63%), myxoid change in 2/55 (3.63%) and multinucleation in 1/55 (1.81%) tumours. One tumour had whorls and small clusters of spindle cells with bland nuclei surrounding the tumour cells, staining strongly for beta-catenin and were negative for pancytokeratin.

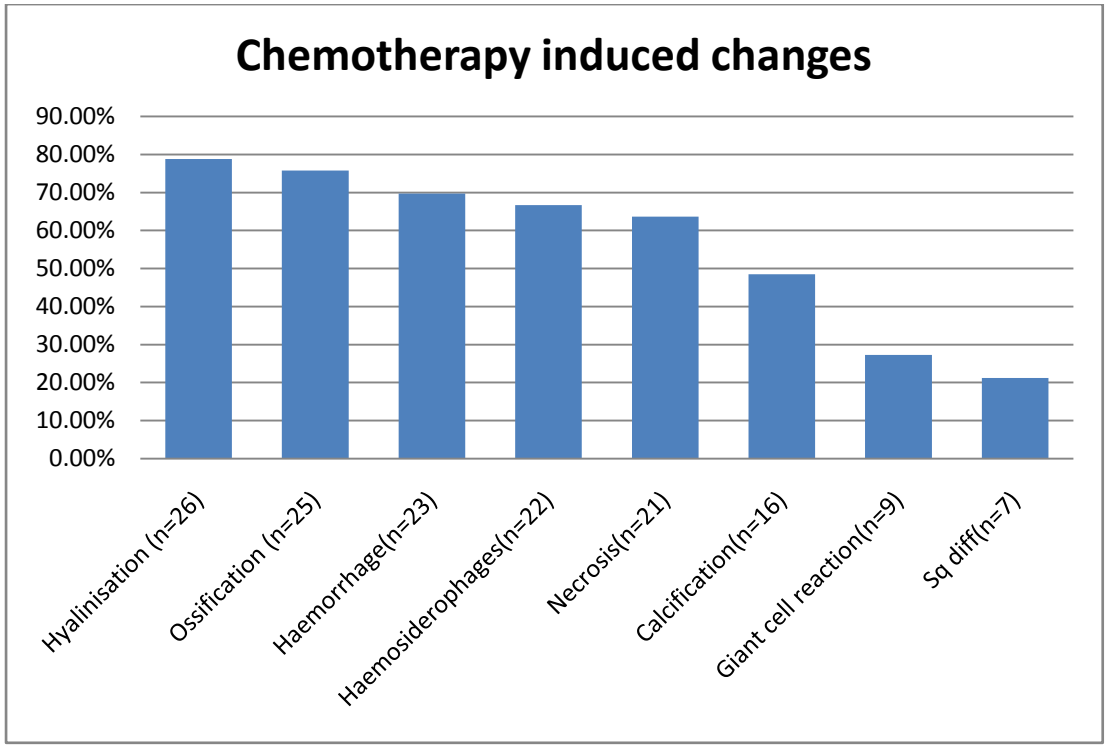


Figure 9: Chemotherapy induced changes in HB (n=33)

Percentage of viable tumour:

About 19/33 (57.58%) patients had ≥50% viable tumour following chemotherapy (Fig 10). Two patients had extensive ossification and no viable tumour. One patient had a few viable islands of normal looking hepatocytes in between the ossified areas constituting approximately 2-5% of the tumour. These areas were highlighted by immunostaining with EpCAM, indicating that these islands were actually tumour cells which could not be picked up initially on H&E staining.

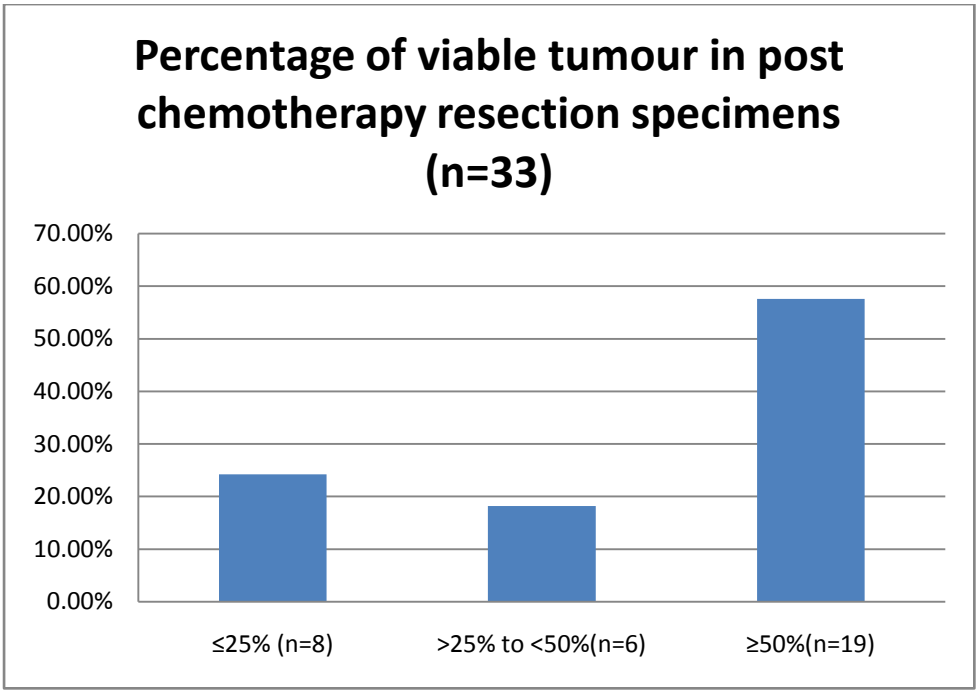


Figure 10: Percentage of viable tumour following chemotherapy in HB(n=33)

Tumour margin:

Of the 31 viable tumours in the post-chemotherapy group, margin could be measured microscopically for 30 tumours, of which 22 (73.34%) had a margin of ≤0.5cm and 4 (13.33%) tumours each with a margin of 0.6-1cm and ≥1cm. When the margin clearance was compared with death, it was found that 6/18 (33.33%) patients with margin ≤0.5cm died of disease (Table 7).

Table 7: Comparison of margin clearance of the tumour with death (n=23):

	No death	**Death**	**P value**
Margin ≤0.5cm	12 (66.67%)	6 (33.33%)	
Margin 0.6-1cm	3 (100%)	0 (0%)	0.41
Margin ≥1cm	1 (50%)	1 (50%)	

Recurrence, metastasis and death:

Recurrence of tumour was found in 3/55 (5.45%) cases, of which two developed recurrent tumour in the same lobe and one in the opposite lobe.

Distant metastasis was documented in 10/55 (18.18%) cases, of which three cases underwent resection of the metastatic tumours in the lung, ileum and omentum respectively. Of the 10 cases, 6 (60%) had metastasis to only one site, the most common being the lung in 5 cases and omentum in the remaining one case. Four cases (40%) had metastasis involving multiple sites like rectovesicle pouch, lymph nodes, diaphragm, mesentery, brain and multiple bones of the skeletal system.

Details about the survival were available for 35/55 patients, of which 15 (42.85%) died due to disease.

Comparison of various parameters in subtypes of HB: Post - chemotherapy group:

There was no significant difference between the various subtypes of HB and MVI, recurrence, metastasis and death(Table 8& Fig 11). However, cases with a predominantly fetal subtype had comparatively decreased chance of MVI, recurrence, metastasis and death. One case with SCUD subtype had MVI, metastasis to lymph node and died due to disease. Of the two cases with focal SCUD areas, MVI was seen in both the tumours and one patient also had metastasis to the lung.

Table 8: Comparison of histologic subtypes with tumour behaviour in HB: Post-chemotherapy group

HB SUBTYPE	MVI (p=0.38)	Recurrence (p=0.82)	Metastasis (p=0.12)	Death (p=0.40)
Predom. fetal	2/8 (25%)	0/8 (0%)	0/8 (0%)	1/5 (20%)
Mixed epithelial	6/11(55%)	2/11(18.18%)	9/11 (81.82%)	2/10 (20%)
MEM	5/11(45.45%)	1/11 (9.09%)	7/11 (63.64%)	4/9 (44.44%)
SCUD	1/1 (100%)	0/1 (0%)	1/1 (100%)	1/1 (100%)

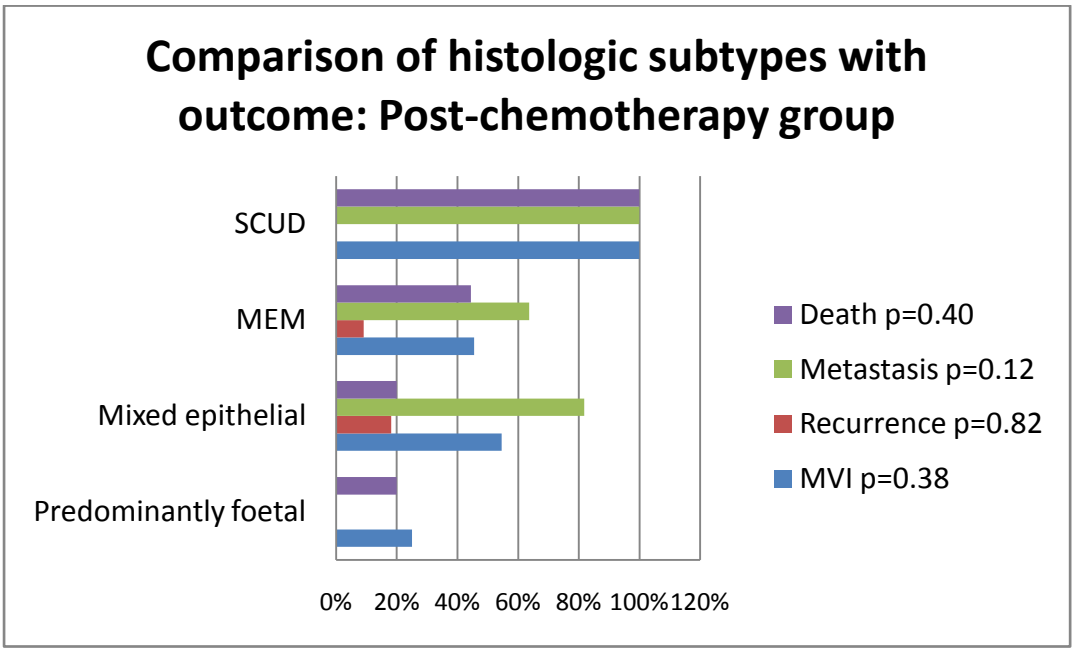
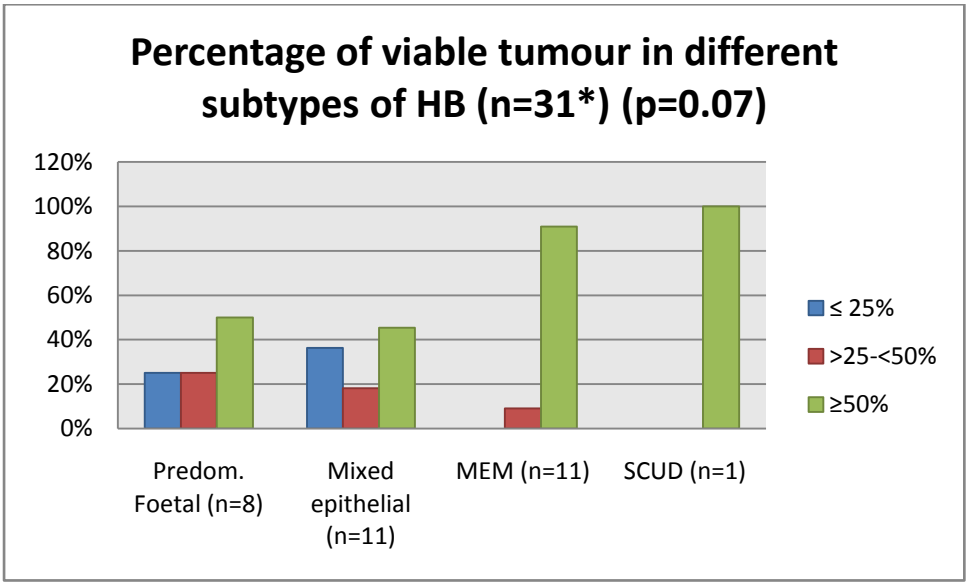


Figure 11: Comparison of histologic subtypes with tumour behaviourin HB: Post-chemotherapy group

Comparison of subtypes of HB with percentage of viable tumour in post-chemotherapy group:

When the percentage of viable tumour was compared with the subtype of HB, it was found that a higher percentage of MEM (90.90%) and SCUD subtypes (100%) had ≥50% viable tumour, however this was not statistically significant (Fig 12).



* Two cases had complete response to chemotherapy and no viable tumour.

Figure 12: Percentage of viable tumour in different subtypes of HB (n=31)

Comparison of PRETEXT with outcome:

There was no significant difference between the different PRETEXT stages when MVI, recurrence and metastasis was compared (Table 9). However, a significant number of patients who belonged to PRETEXT III and IV succumbed to the disease (Fig 13).

Table 9: Comparison of PRETEXT with outcome in HB:

	PRETEXT I (n=3)	PRETEXT II (n=13)	PRETEXT III (n=6)	PRETEXT IV(n=3)	p value
MVI	1 (33.33%)	5 (38.46%)	3 (50%)	0 (0%)	0.54
Recurrence	1 (33.33%)	0 (0%)	2 (33.33%)	0 (0%)	0.10
Metastasis	2 (66.66%)	2 (15.38%)	2 (33.33%)	1 (33.33%)	0.24
Death	2 (66.66%)	1 (7.69%)	5 (83.33%)	3 (100%)	<0.001*

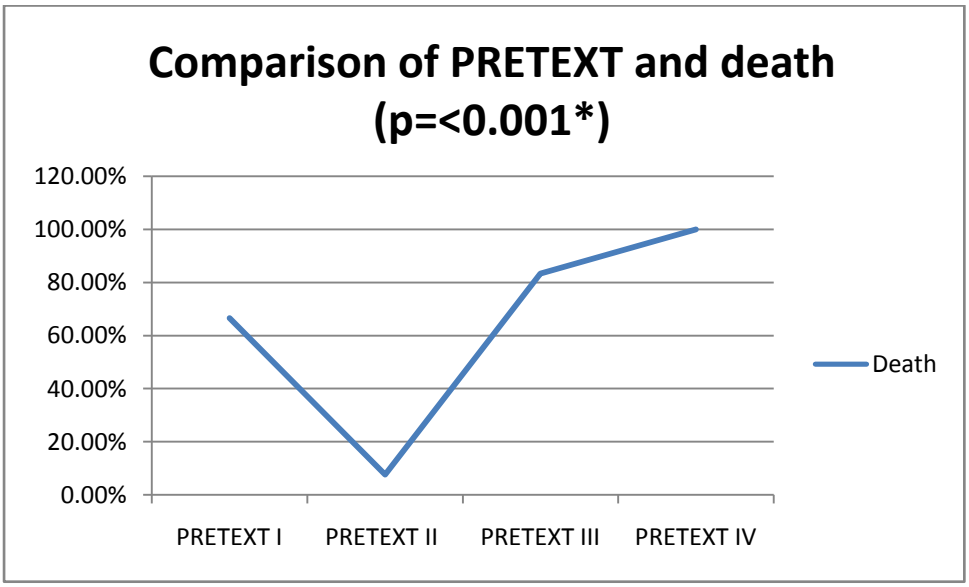


Figure 13: Comparison of PRETEXT with death in HB patients (n=25)

Immunohistochemical expression of CK19, Beta-catenin and EpCAM:

Out of the total 44 pre-chemotherapy samples, 37 blocks were available for IHC analysis. Similarly in the post-chemotherapy group, a total of 30 blocks were available for IHC. Of this, two patients had complete response to chemotherapy with no viable tumour. Hence IHC was carried out on 28 patients in this group.

Expression of CK-19:

In the pre-chemotherapy group, out of 37 cases, 32 tumours had fetal and 24 had embryonal components either alone or in combination. CK19 expression was observed only in 7/32 (21.88%) tumours, in the fetal component, whereas in the embryonal, it was found in 13/24 (54.17%) tumours and the difference between the two types was statistically significant (p= 0.01*).

In the post-chemotherapy group, out of 28 cases, fetal and embryonal components were seen in 23 and 18 tumours respectively. CK19 expression was seen in 4/23 (17.39%) fetal and 13/18 (72.22%) embryonal components and the difference between the two types was statistically significant (p=<0.001*) (Fig 14)

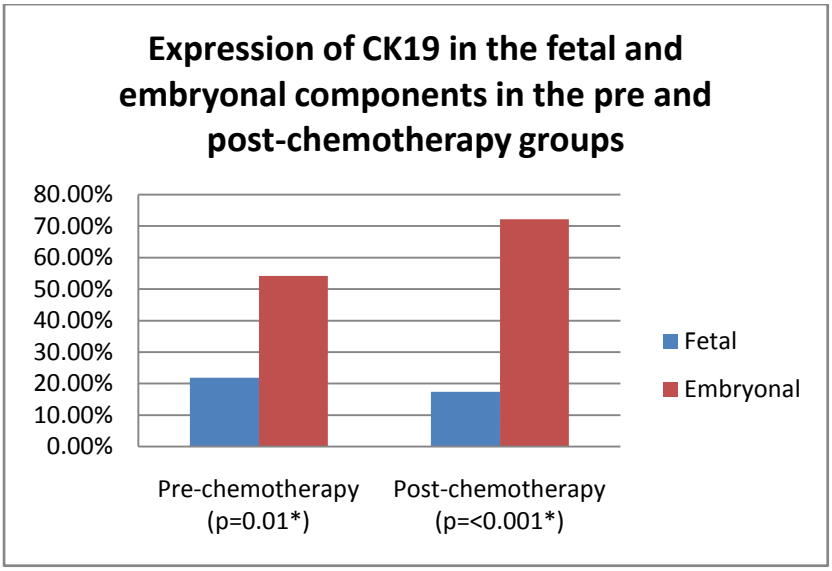


Figure 14: Expression of CK19 in fetal and embryonal components in pre and post-chemotherapy groups

Percentage of CK19 positive tumour cells in the fetal and embryonal components in the pre and post chemotherapy groups:

In the pre-chemotherapy group,6/13 (46.15%) tumours with embryonal component had >50% of cells staining for CK-19, whereas in the fetal subtype, 6/7 (85.71%) tumours had <25% of cells showing CK-19 expression and the difference was statistically significant (p=0.04*). In the post-chemotherapy group, although7/13 (53.84%) tumours with embryonal component had >50% cells staining for CK-19, when compared to 1/4 (25%) tumours in the fetal subtype, there was no statistically significant difference between the two groups(p=0.34)(Fig 15).

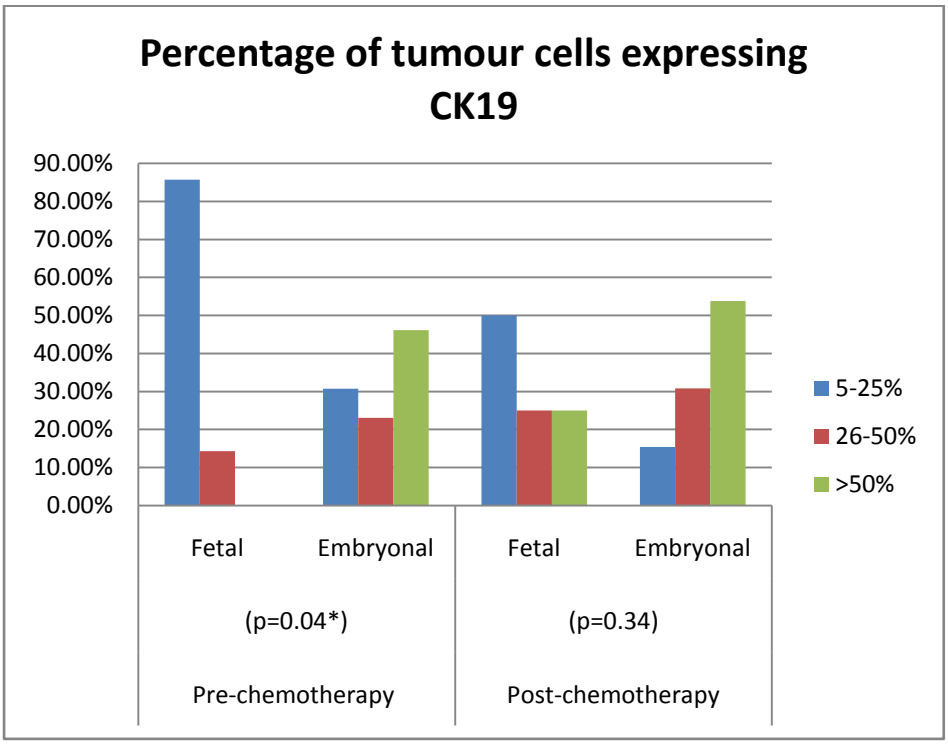


Figure 15: Percentage of CK19 positive tumour cells in the fetal and embryonal components of HB in pre and post chemotherapy groups

Intensity of expression of CK19 in the fetal and embryonal components:

When the intensity of expression was compared between the fetal and the embryonal components there was no significant difference in both the pre and post-chemotherapy groups (Table 10).

Table 10: Intensity of CK19 expression in the fetal and embryonal components of HB, in the pre and post-chemotherapy groups:

	Pre-chemotherapy (p=0.79)		**Post-chemotherapy (p=0.54)**	
	Fetal (n=7)	Embryonal(n=13)	Fetal (n=4)	Embryonal(n=13)
Moderate	2 (28.57%)	3 (23.08%)	0 (0.00%)	3 (23.08%)
Strong	5 (71.43%)	10 (76.92%)	4 (100.00%)	10 (76.92%)

Beta-catenin:

Nuclear± cytoplasmic (N/ N+C) expression was seen in 18/37 (48.65%) and 16/28(57.14%) of tumours in the pre and post-chemotherapy groups,respectively (Fig 16).

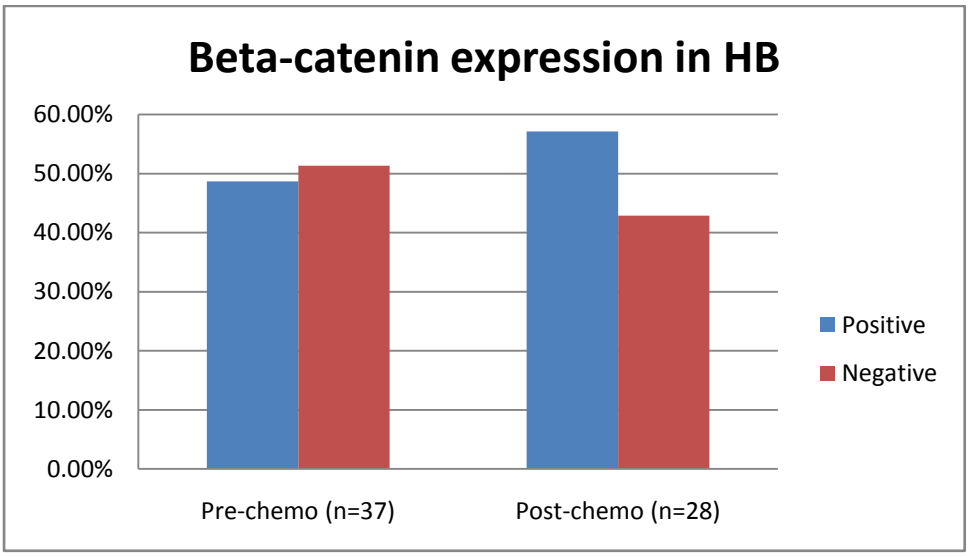


Figure 16: Expression of Beta-Catenin in the pre and post-chemotherapy groups

Intensity and percentage of cells showing nuclear expression of beta-catenin:

Majority of the tumours, 13/18 (72.22%) and 8/16 (50%) in the pre and post chemotherapy groups respectively,expressed beta catenin in only 5- <25% of cells. Three tumours in each group (16.67% and 18.75%) had >50% of cells expressing the marker and the remaining showed expression in 25-50% of cells (Fig 17).

Nuclear expression of beta-catenin was strong in a majority of the tumours – 16/18 (88.88%) and 14/16 (87.50%) in the pre and post-chemotherapy groups respectively. In the remaining two cases in each group, beta catenin expression was moderate in intensity (Fig 18).

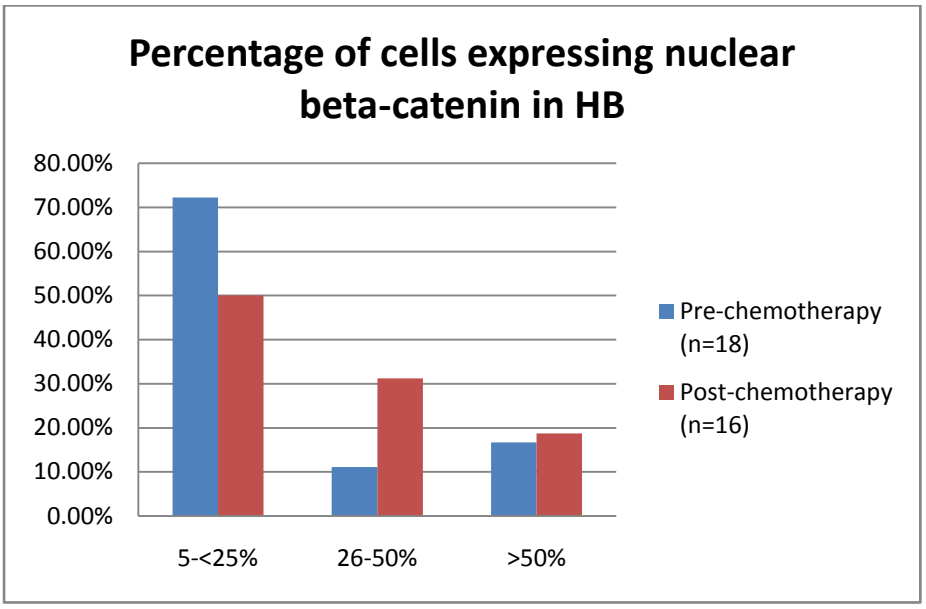


Figure 17: Percentage of cells expressing nuclear beta-catenin in the pre and post-chemotherapy groups

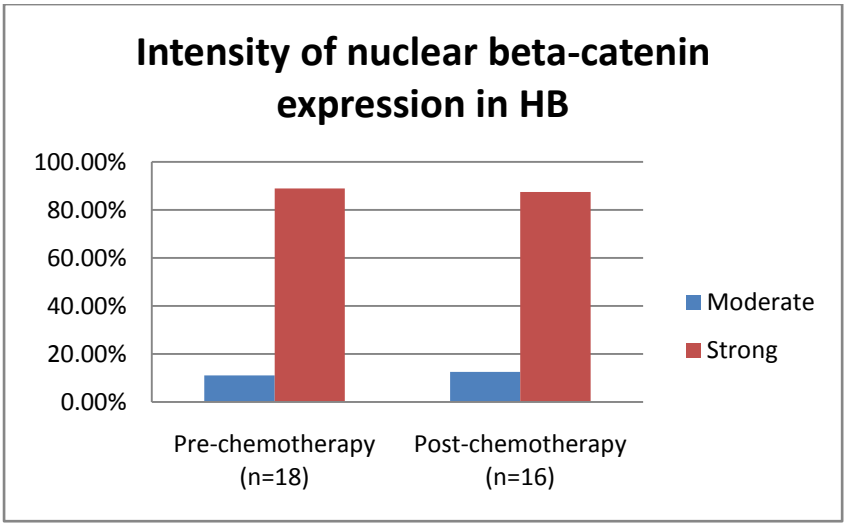


Figure 18: Intensity of nuclear expression of beta-catenin in the pre and post chemotherapy groups

EpCAM:

EpCAM was expressed in all the tumours 37/37(100%) in the pre-chemotherapy group and in 23/28 (82.14%) tumours in the post-chemotherapy group. Majority of the tumours 34/37 (91.90%) and 19/23 (82.62%) in the pre and post-chemotherapy groups respectively, showed intense expression of EpCAM. Weak expression of EpCAM was seen in 1/37 (2.70%) and 2/23 (8.69%) tumours in the pre and post chemotherapy groups respectively, while the remaining showed moderate expression (Fig 19).

One case had good response to chemotherapy with predominantly ossified areas and few viable clusters of cells which resembled normal hepatocytes on H&E section. However, EpCAM highlighted these viable clusters, thereby identifying them as residual tumour clusters.

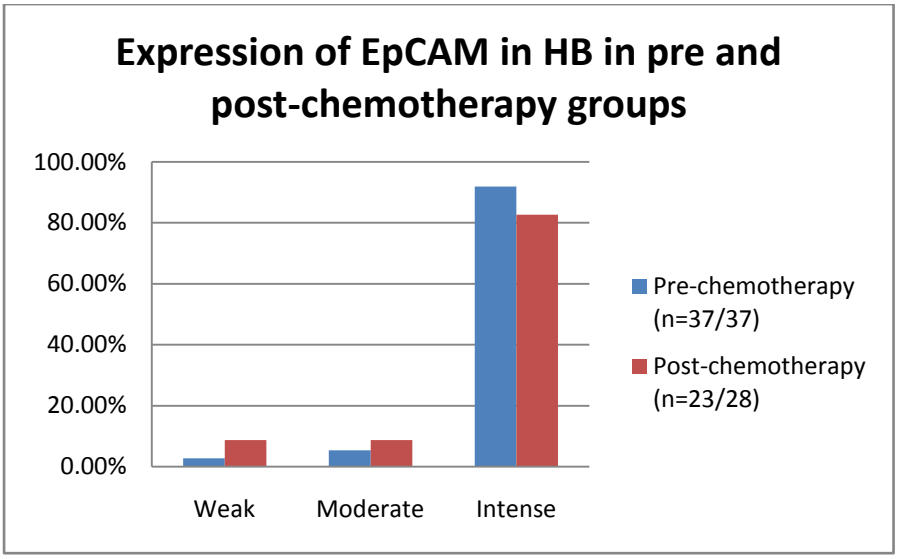


Figure 19: Expression of EpCAM in the pre and post chemotherapy groups

Comparison of immunohistochemical marker expression and tumour behaviour:

There was no significant difference in parameters of tumour behaviour like MVI, recurrence, metastasis and death when the immunohistochemical expression of CK19 (Table 11&12), Beta-catenin (Table 13) and EpCAM (Table 14) were compared.

Table 11: Comparison of CK19 expression and tumour behaviour in the post-chemotherapy group - Fetal component (n=23):

		CK19 –ve (n=19)	CK19 +ve (n=4)	P value
MVI	Absent	11 (57.89%)	2 (50%)	0.77
	Present	8 (42.11%)	2 (50%)	
Recurrence	Absent	17 (89.47%)	4 (100%)	0.49
	Present	2 (10.53%)	0 (0%)	
Metastasis	Absent	15 (78.95%)	4 (100%)	0.31
	Present	4 (21.05%)	0 (0%)	
Death*	Absent	11 (73.33%)	2 (50%)	0.37
	Present	4 (26.67%)	2 (50%)	

* Details about death were not available for 4 patients with absent CK19 expression

Table 12: Comparison of CK19 expression and tumour behaviour in the post-chemotherapy group –Embryonal component (n=18):

		CK19 –ve (n=5)	CK19 +ve (n=13)	P value
MVI	Absent	1 (20%)	7 (53.85%)	0.19
	Present	4 (80%)	6 (46.15%)	
Recurrence	Absent	4 (80%)	11 (84.62%)	0.81
	Present	1 (20%)	2 (15.38%)	
Metastasis	Absent	5 (100%)	7 (53.85%)	0.31
	Present	0 (0%)	6 (46.15%)	
Death*	Absent	3 (75%)	7 (58.33%)	0.55
	Present	1 (25%)	5 (41.67%)	

* Details about death were not available for 1 patient with absent CK19 expression

Table 13: Comparison of Beta-catenin expression and tumour behaviour in the post-chemotherapy group (n=25):

		Beta-catenin –ve(n=9)	Beta-catenin +ve (n=16)	P value
MVI	Absent	5 (55.56%)	9 (56.25%)	0.64
	Present	4 (44.44%)	7 (43.75%)	
Recurrence	Absent	9 (100%)	13 (81.25%)	0.24
	Present	0 (0%)	3 (18.75%)	
Metastasis	Absent	7 (77.78%)	13 (81.25%)	0.60
	Present	2 (22.22%)	3 (18.75%)	
Death*	Absent	4 (66.67%)	10 (66.67%)	0.68
	Present	2 (33.33%)	5 (33.33%)	

Death details were available only for 21 patients (Beta-catenin -ve n=6; Beta-catenin +ve n=15)

Table 14: Comparison of EpCAM expression and tumour behaviour in the post-chemotherapy group (n=28):

		EpCAM –ve (n=5)	EpCAM +ve (n=23)	P value
MVI	Absent	3 (60%)	12 (52.17%)	0.75
	Present	2 (40%)	11 (47.83%)	
Recurrence	Absent	5 (100%)	20 (86.96%)	0.39
	Present	0 (0%)	3 (13.04%)	
Metastasis	Absent	4 (80%)	17 (73.91%)	0.77
	Present	1 (20%)	6 (26.09%)	
Death*	Absent	2 (50%)	14 (70%)	0.43
	Present	2 (50%)	6 (30%)	

*Death details were available only for 24 patients (EpCAM -ve group n=4; EpCAM +ve group n=20)

Expression of EpCAM with percentage of viable tumour:

When the percentage of viable tumour was correlated with the intensity of expression of EpCAM, 93.93% of the cases with intense expression had ≥50% viable tumour (p=0.04*) (Table 15/ Fig 20).

Table 15: Comparison of percentage of viable tumour with expression of EpCAM:

Percentage of viable tumour	Weak	Moderate	Intense	P value
≤25%	0 (0%)	2 (50%)	2 (50%)	0.04*
>25%-<50%	1 (25%)	0 (0%)	3 (75%)	
≥50%	1 (6.67%)	0 (0%)	14 (93.93%)	

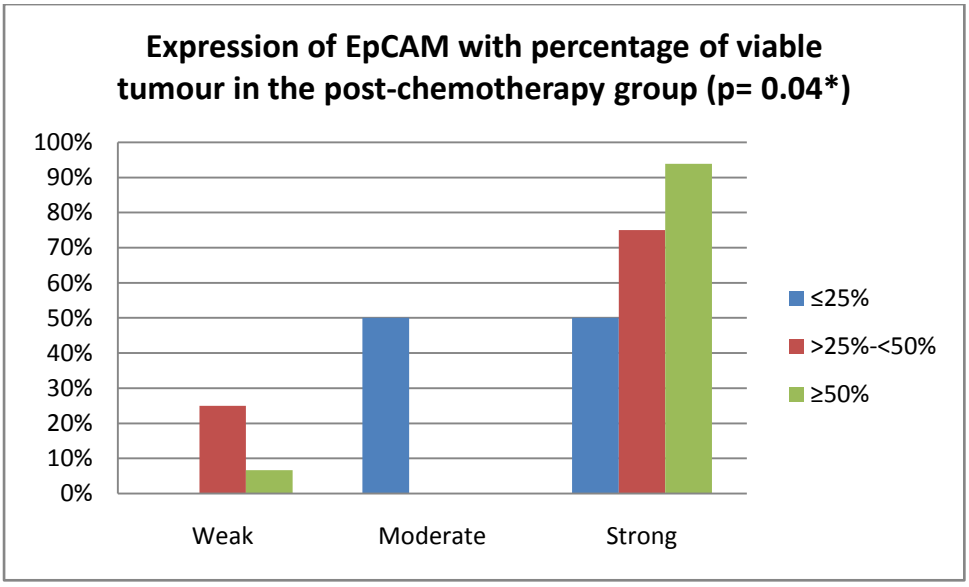


Figure 20: Expression of EpCAM with percentage of viable tumour (n=23)

Survival analysis:

A total of 22 patients for whom the follow up details were available were taken for analysis. The overall median (range) survival was 70.71(1.65- 109.90) months. The mean EFS in our study was 59.49 months (95% CI: 35.71 – 83.28) (Fig 21). The five year OS was 60%.

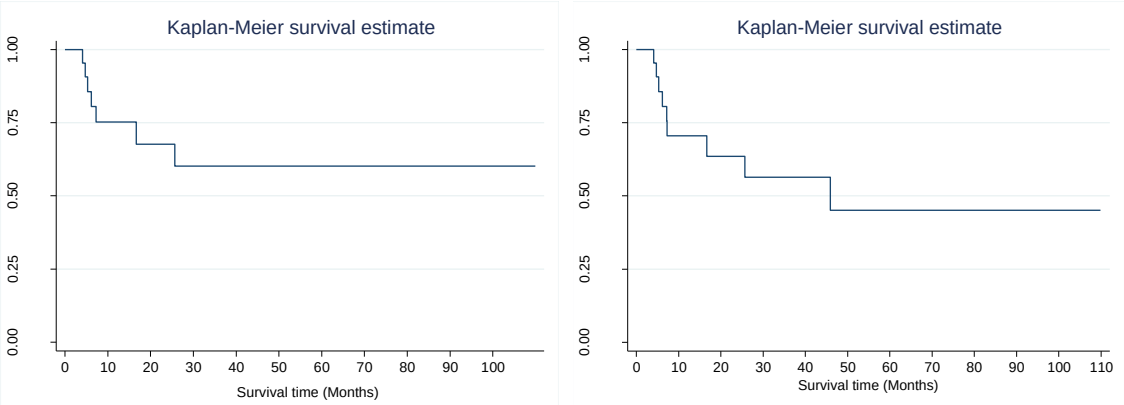


Figure 21: Kaplan Meier survival graphs – Overall and Event free survival

Kaplan-Meier survival curve for metastasis free survival:

The patients who developed metastasis (n=10) are plotted in the graph. All events occurred within 50 months after diagnosis. At 109 months (9 .08 years) of follow-up, the curve indicates an estimated freedom from metastases of 56.5% (95% confidence interval, 34.5%-76.8%) (Fig 22)

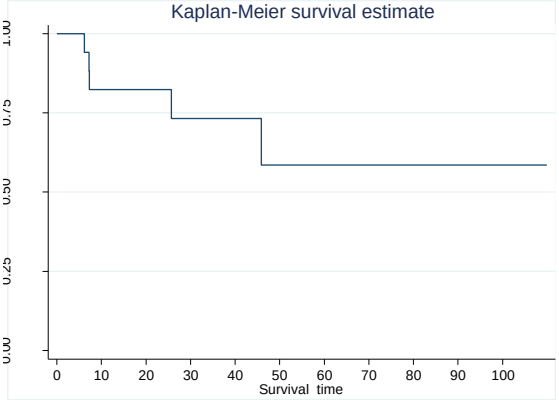


Figure 22: Kaplan Meier survival graph for metastasis free survival

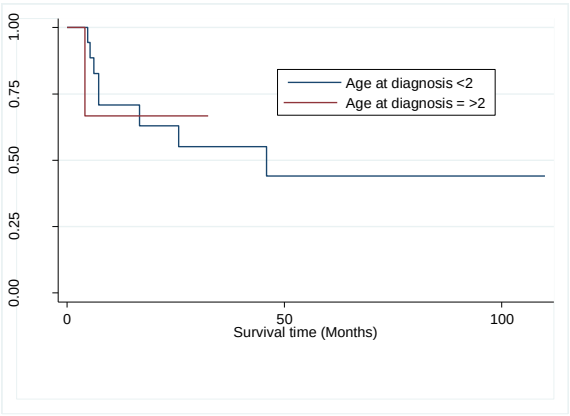
Kaplan Meier survival estimates for event free survival:

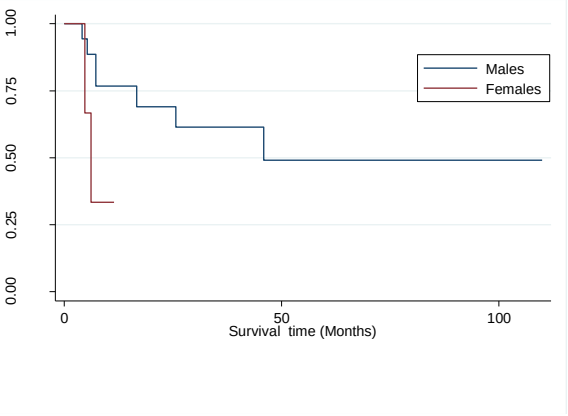
There was a statistically significant difference in the event free survival (EFS) between the patients who presented with a solitary tumour nodule when compared to multiple nodules (p=0.001) and patients with <50% viable tumour when compared to ≥50% viable tumour following chemotherapy (p=0.04). However, other factors like age at diagnosis ≤2yrs, male sex, AFP levels following <10000 IU/ml following chemotherapy, tumour dimension ≤5cm, PRETEXT I&II, mitosis ≤2/10hpf and absent nuclear expression of beta-catenin had higher EFS rates (Table 16/Fig 23).

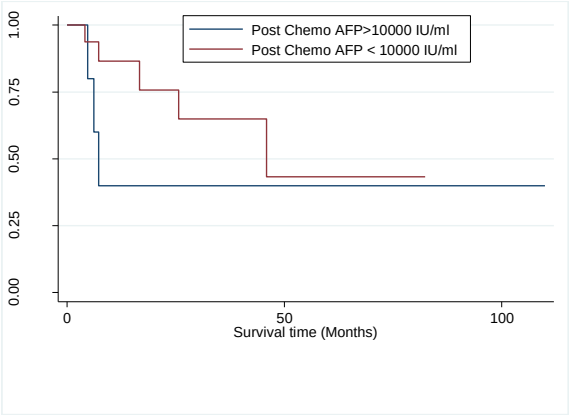
The overall survival for patients without a lung metastasis was 75.86 months when compared to 28.38 months for those with lung metastasis.

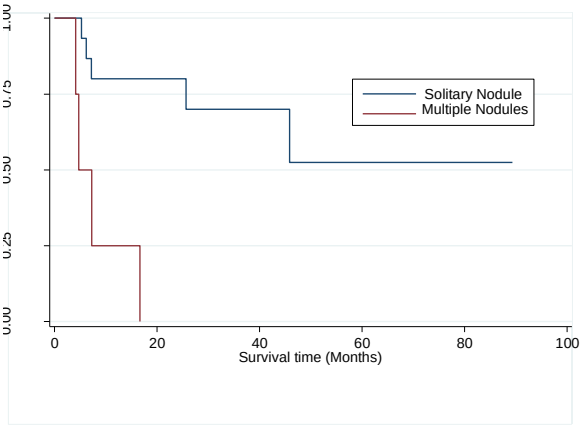
Table 16: EFS of HB patients (n=23):

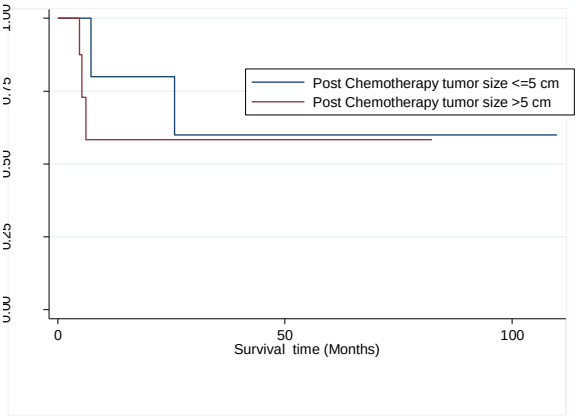
Parameters		Mean survival (in months)	Events (No.)	95% C.I	P value
Age at diagnosis	≤2yrs	58.62	8	33.68 - 83.56	
	>2yrs	22.97	1	7.87 - 38.08	0.94
Sex	Males	64.25	7	39.16 - 89.35	
	Females	7.45	2	4.18 - 10.71	0.09
Post-chemoAFP	<10000 IU/ml	50.92	5	30.78 - 71.06	
	>10000 IU/ml	47.58	3	2.98 - 92.19	0.34
No of lesions	Solitary	58.77	5	37.85 - 79.69	
	Multiple	8.20	4	3.26 - 13.13	0.001*
Tumour size	≤5cm	72.53	2	32.09 – 112.97	
	>5cm	50.30	3	22.33 -78.26	0.67
PRETEXT	I&II	63.89	3	41.15 - 86.62	
	III&IV	43.77	2	-9.42 - 96.97	0.23
MVI	Absent	72.83	1	55.36 -90.29	
	Present	46.23	7	18.41 - 74.05	0.08
Mitotic count	≤2/10hpf	88.56	1	54.43 -122.71	
	>2/10hpf	28.89	6	4.13 – 53.65	0.10
Viable tumour	<50%	109.90	0	109.90 - 109.90	
	≥ 50%	39.28	8	18.35 - 60.20	0.04*
Nuclear beta-catenin	Absent	66.69	2	22.02 – 111.36	
	Present	45.07	5	18.71 – 71.44	0.67

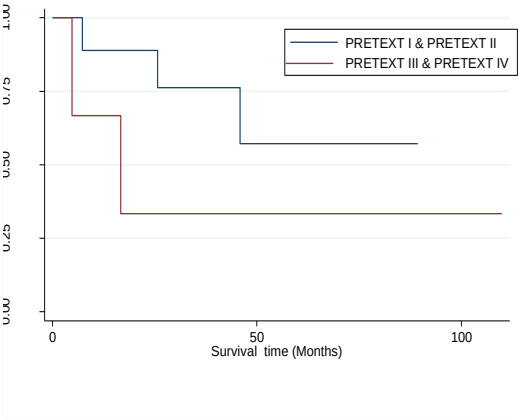

Age at diagnosis <2
Age at diagnosis = >2
0.00
0.25
0.50
0.75
1.00
0
50
100
Survival time (Months)


Males
Females
0.00
0.25
0.50
0.75
1.00
0
50
100
Survival time (Months)


Post Chemo AFP>10000 IU/ml
Post Chemo AFP < 10000 IU/ml
0.00
0.25
0.50
0.75
1.00
0
50
100
Survival time (Months)


Solitary Nodule
Multiple Nodules
0
20
40
60
80
100
Survival time (Months)


Post Chemotherapy tumor size <=5 cm
Post Chemotherapy tumor size >5 cm
0
50
100
Survival time (Months)


PRETEXT I & PRETEXT II
PRETEXT III & PRETEXT IV
0
50
100
Survival time (Months)

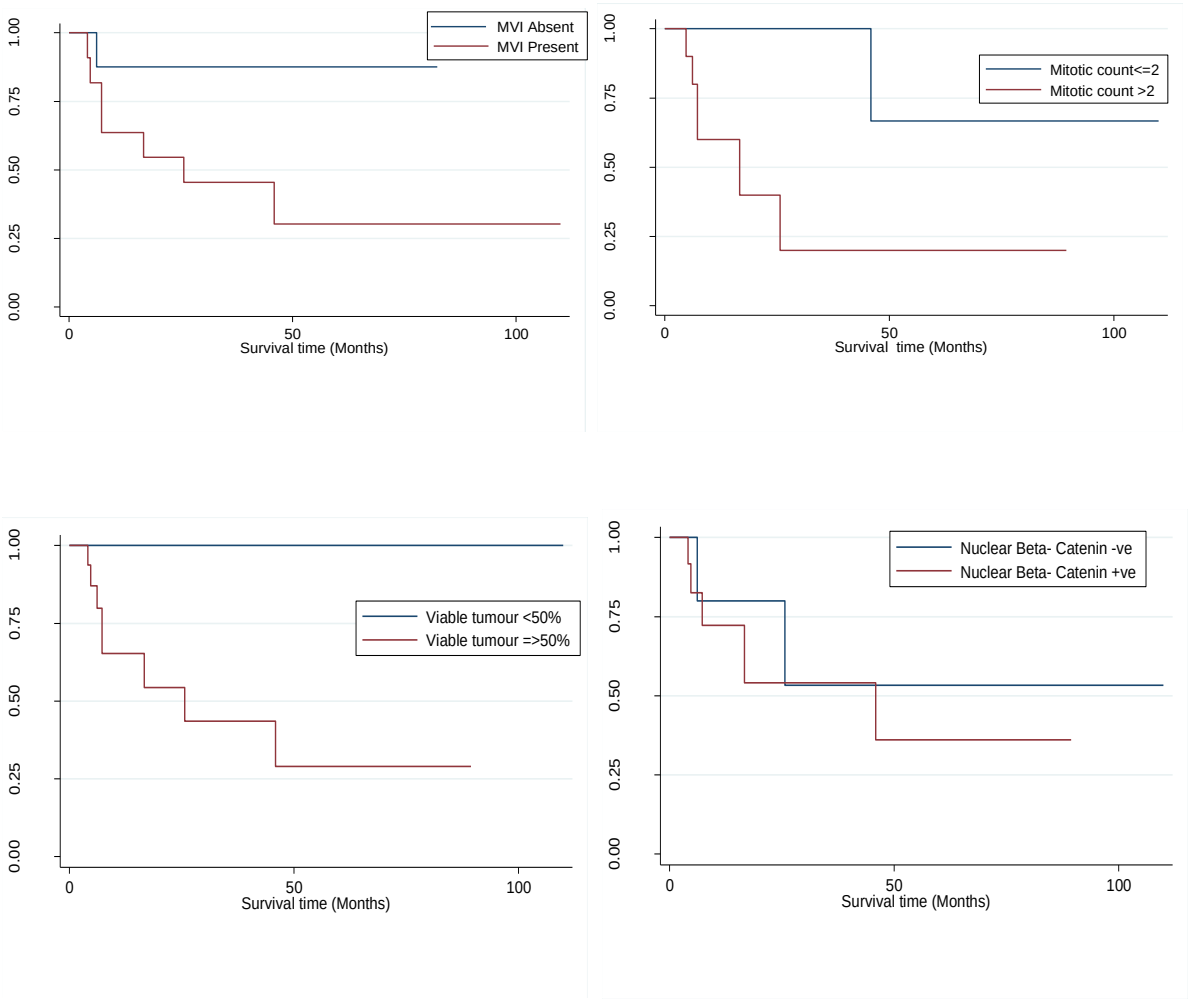


Figure 23: Kaplan Meier survival estimates

ILLUSTRATIONS

GROSS

Figure 1: HB - Variegated yellowish orange to grey white cut surface and with areas of haemorrhage.

Figure 2: Multinodular HB - Grey white to yellowish orange cut surface.

Figure 3: HB - Trabeculated and haemorrhagic cut surface.

Figure 4: HB - Vaguely lobulated tumour with a soft greenish bile stained cut surface.

Figure 5: HB SCUD subtype - Large, lobulated fleshy tumour with grey white cut surface and areas of myxoid degeneration.

Figure 6: Teratoid HB - Bulging grey white firm cut surface with glistening chondro-myxoid areas.

Figure 7: HB with extensive ossification.

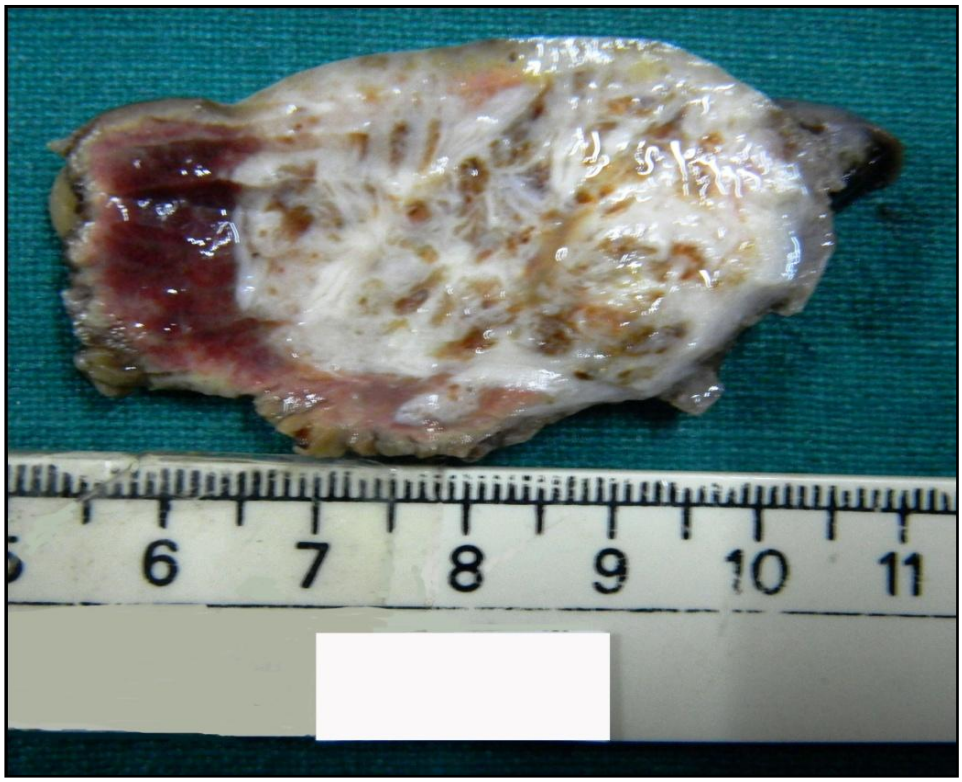


Figure 8: HB with extensive hyalinisation.

Figure 9: Recurrent HB: Multinodular tumour with a tan cut surface and with foci of haemorrhage and necrosis.

Figure 10: Metastatic HB in ileum - Nodular tumour with a grey white cut surface.

MICROSCOPY

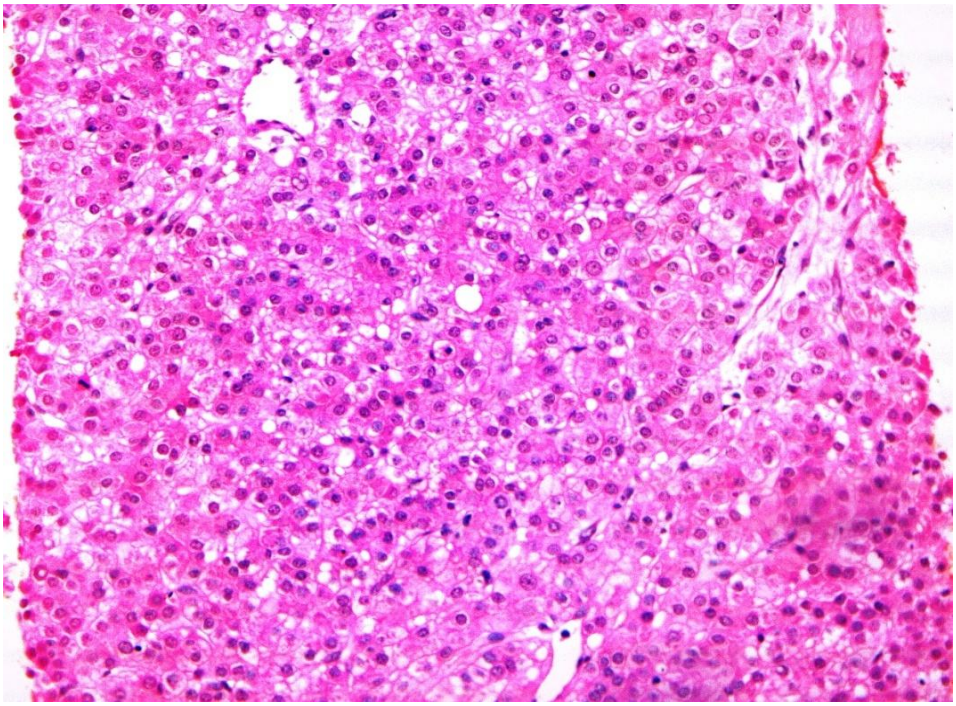


Figure 1: HB – Pure fetal subtype, **H&E 200X**

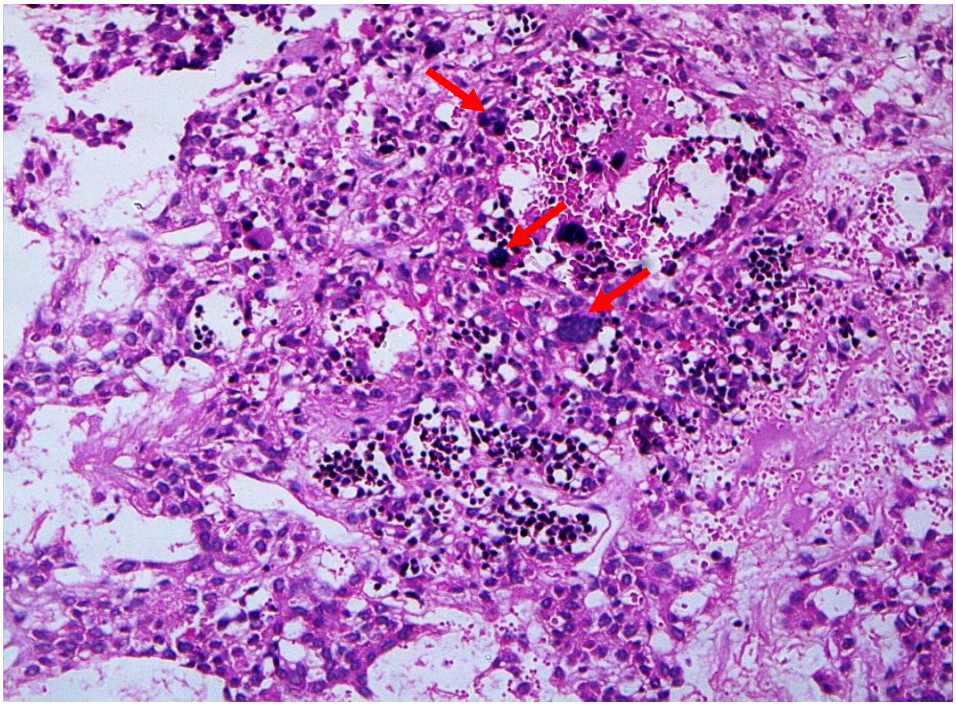


Figure 2: HB - Fetal subtype with foci of extramedullary haematopoiesis including megakaryocytes (arrows), **H&E 100X**

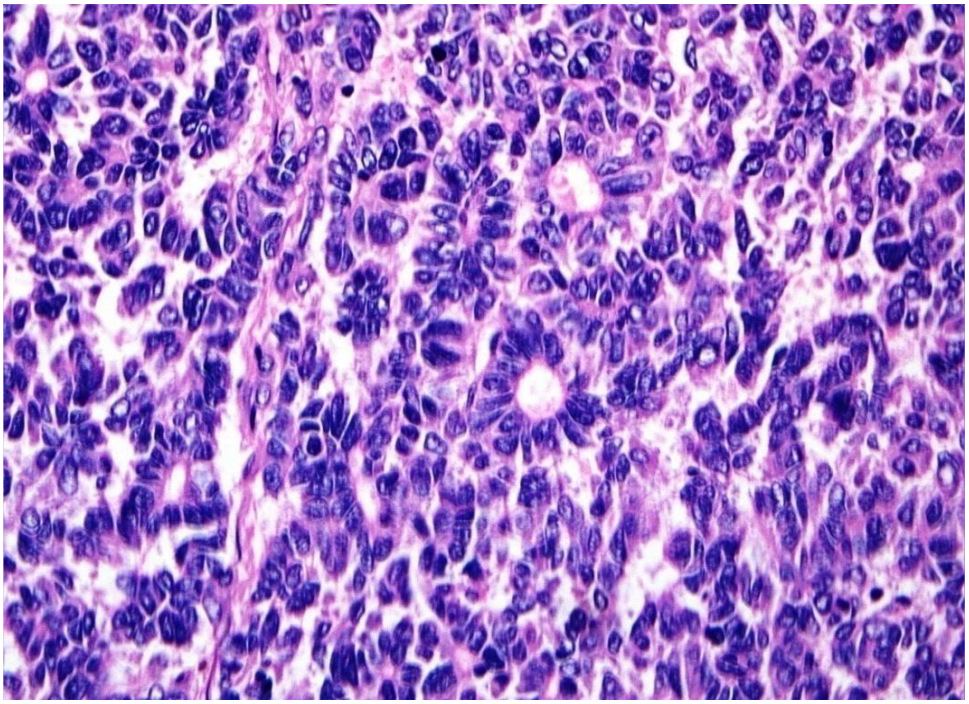


Figure 3: HB - Embryonal subtype with pseudoglandular arrangement of tumour cells, H&E **400X**

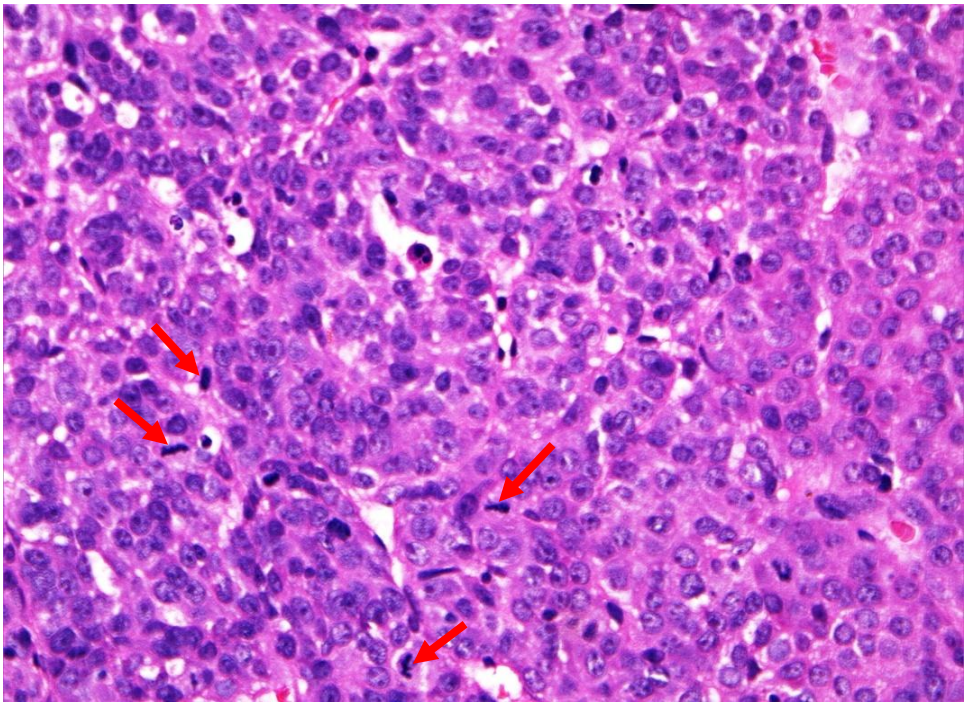


Figure 4: HB - Embryonal subtype with brisk mitotic activity (arrows), H&E **400X**

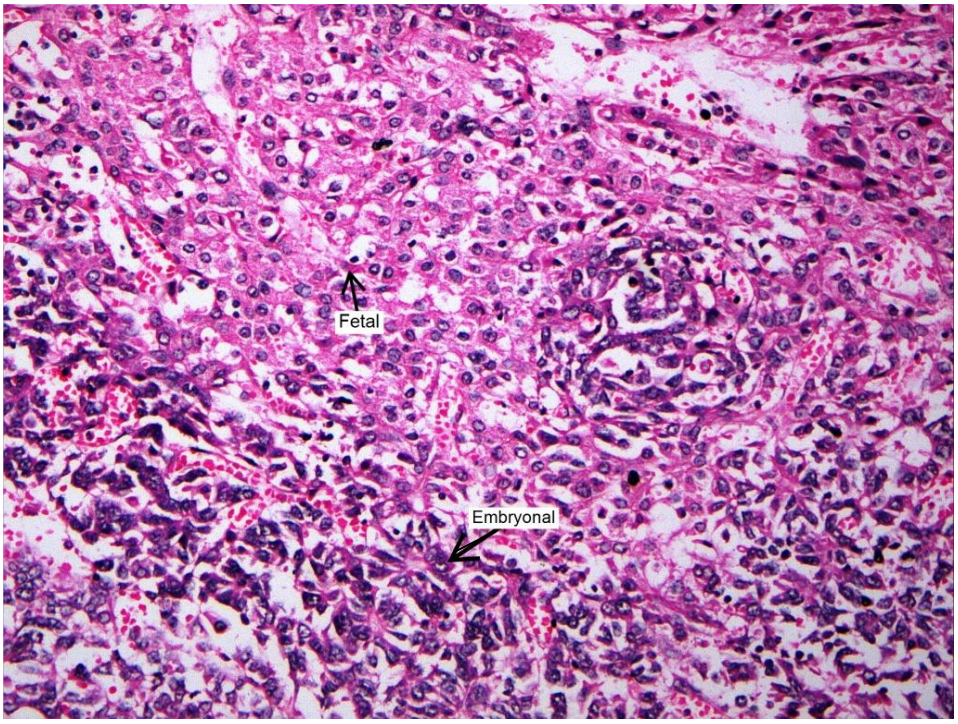


Figure 5: HB - Mixed fetal and embryonal subtypes, H&E **200X**

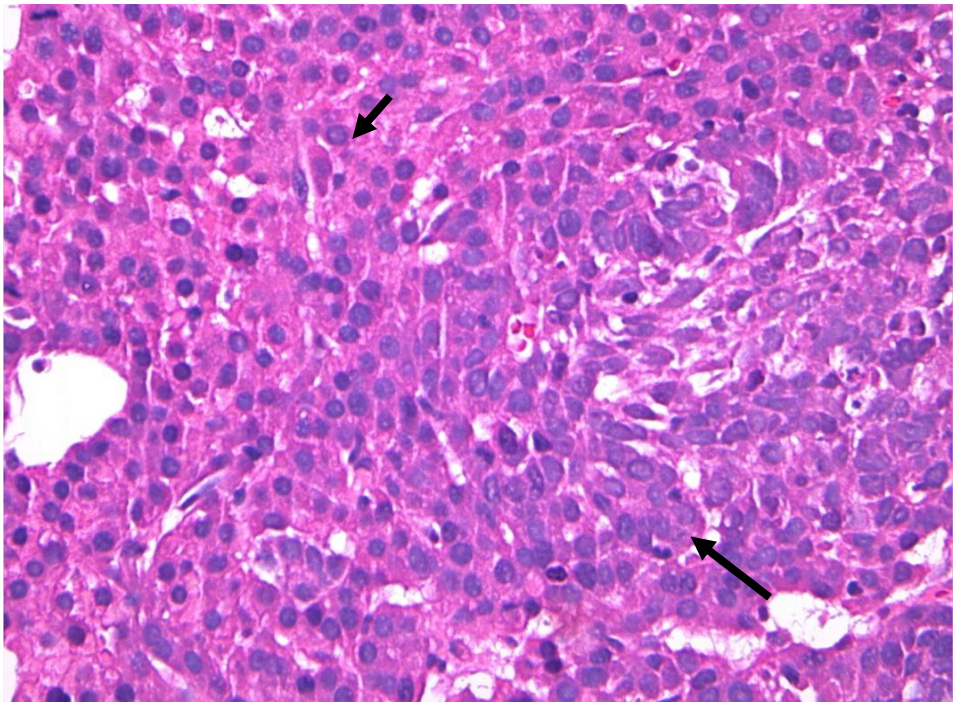


Figure 6: HB - Mixed fetal (short arrow) and embryonal (long arrow) subtype, H&E **400X**

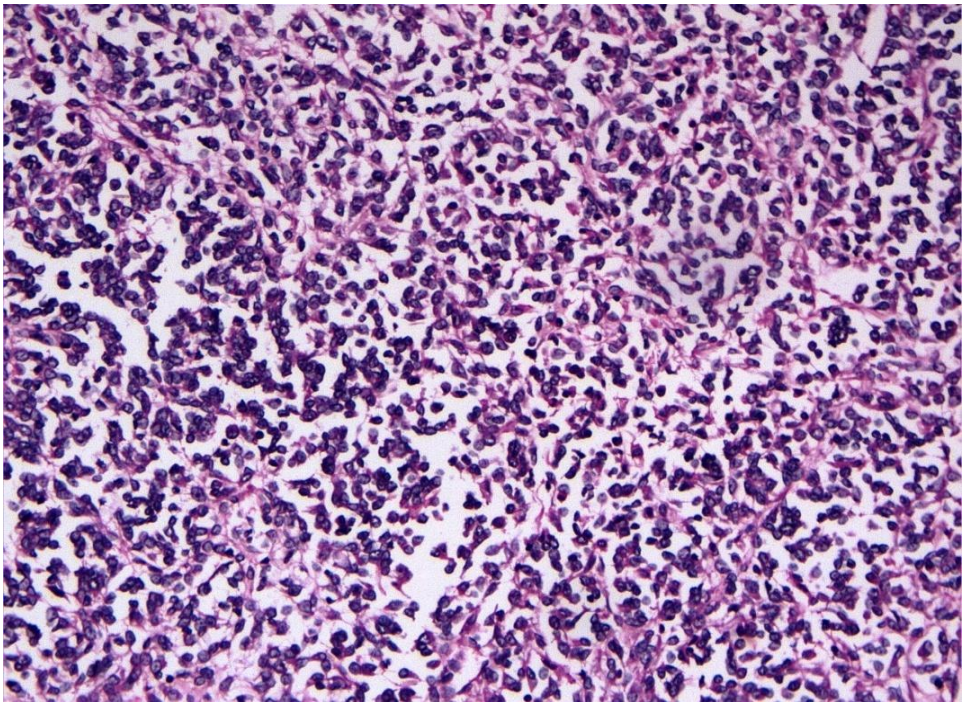


Figure 7: HB - SCUD subtype, sheets of small cells with hyperchromatic nuclei, H&E **200X**

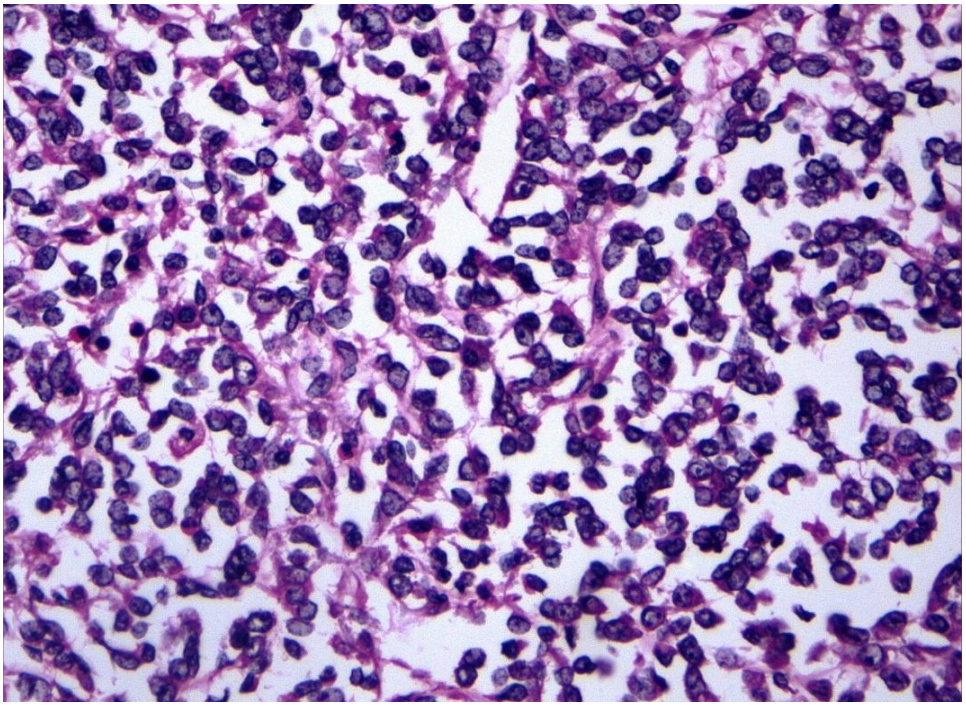


Figure 8: HB - SCUD subtype, high power, H&E **400X**

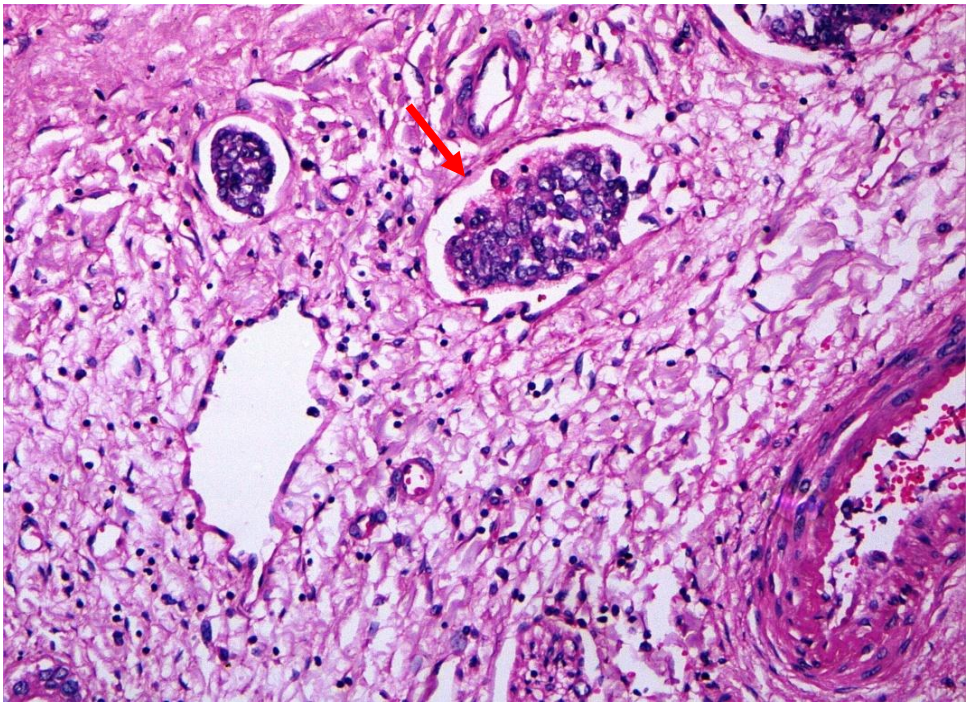


Figure 9: HB - SCUD subtype, microvascular invasion, H&E **200X**

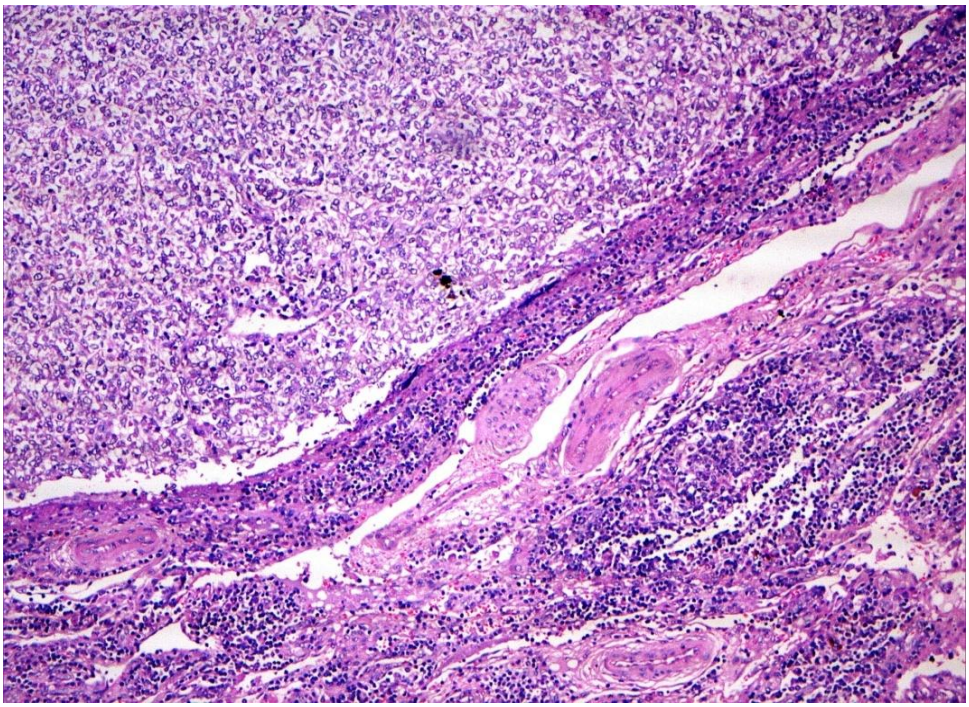


Figure 10: HB - SCUD subtype, lymph node metastasis, same case as above, H&E **10X**

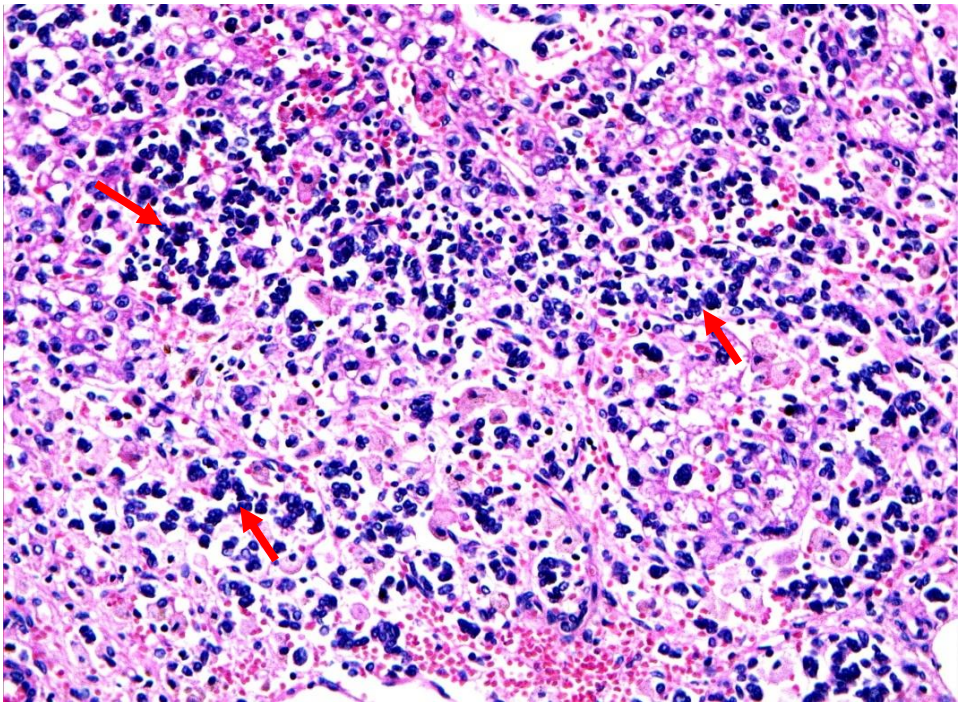


Figure 11: HB - Mixed epithelial subtype with focal SCUD component (arrows), H&E **200X**

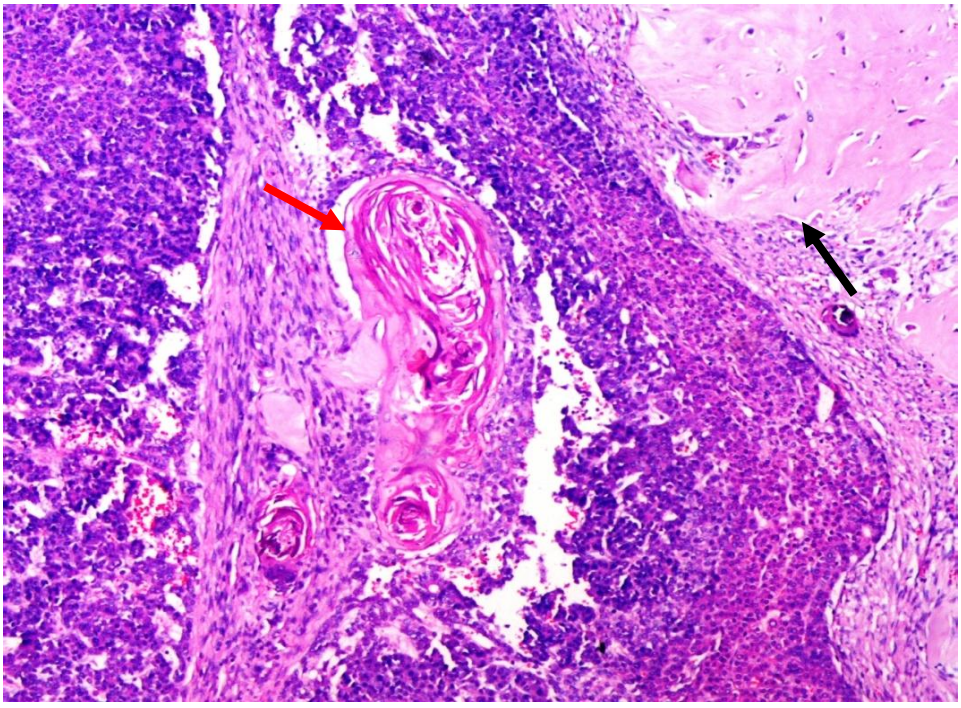


Figure 12: HB - Mixed epithelial and mesenchymal subtype with osteoid (black arrow) and squamous differentiation (red arrow), H&E **100X**

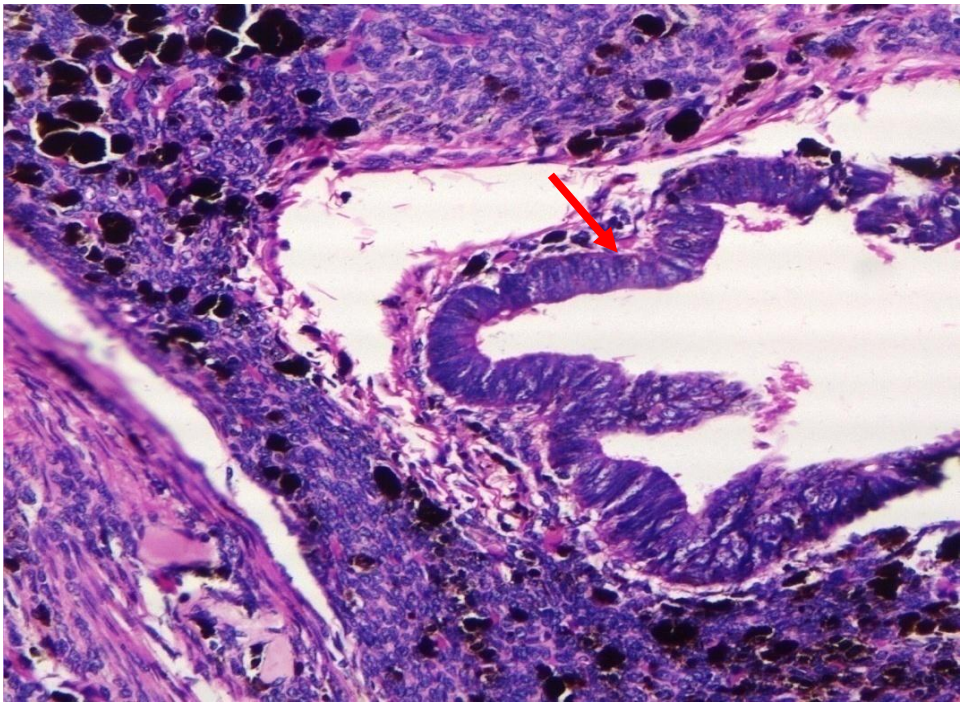


Figure 13: HB -Teratoid subtype with neuroepithelial like structures (arrow) and melanin pigment, H&E **200X**

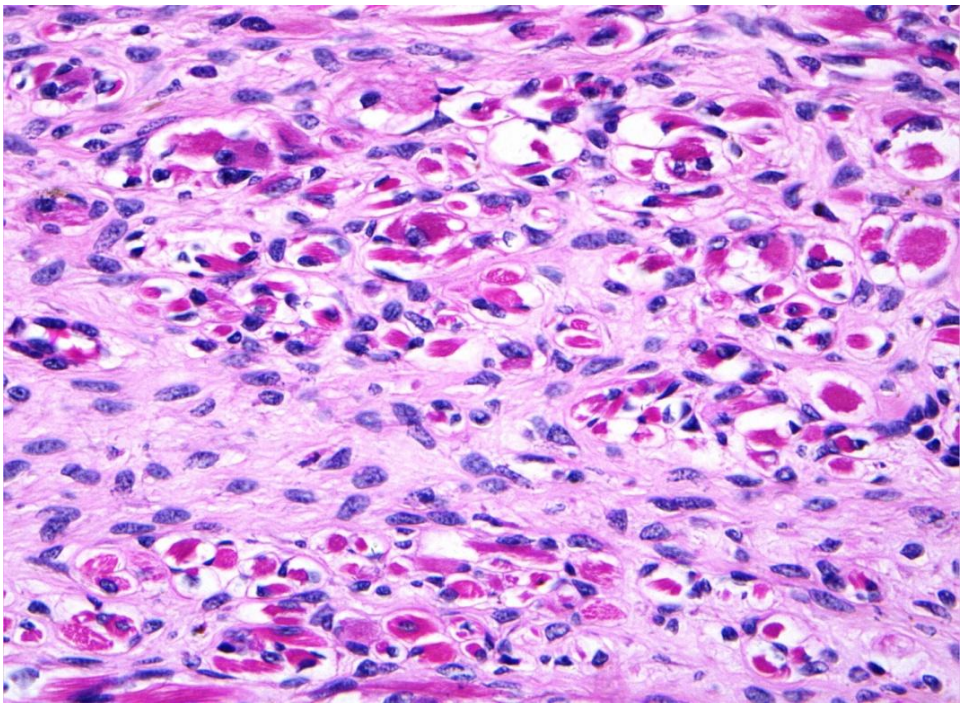


Figure 14: HB -Teratoid subtype with striated muscle fibres, same case as above, H&E **400X**

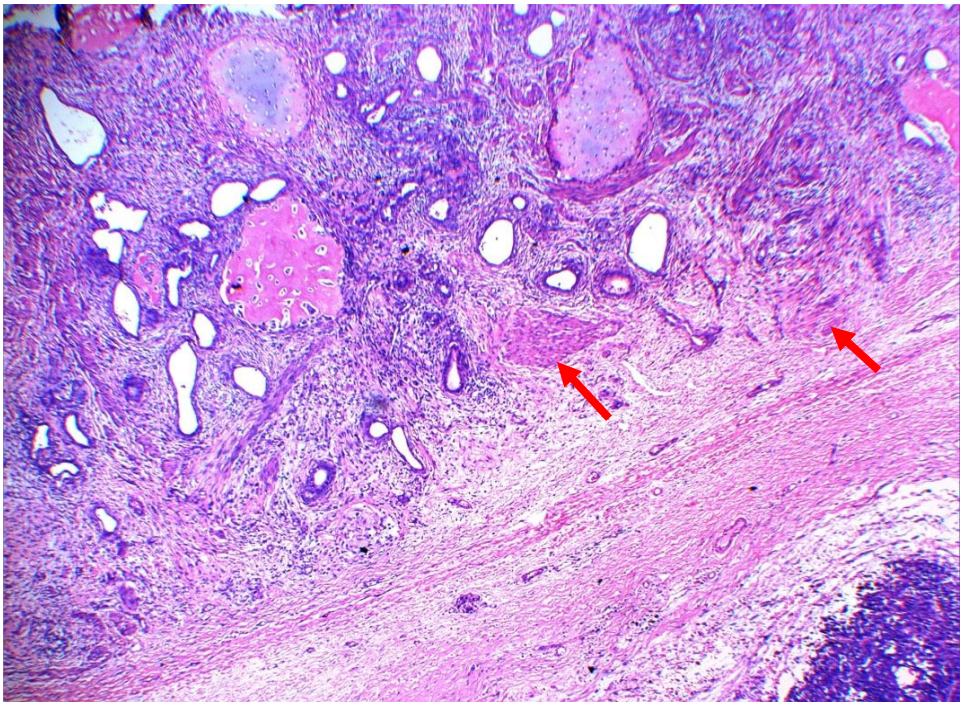


Figure 15: HB - Teratoid subtype with cartilage, bone, immature mesenchyme and smooth muscle bundles (arrows), H&E **40X**

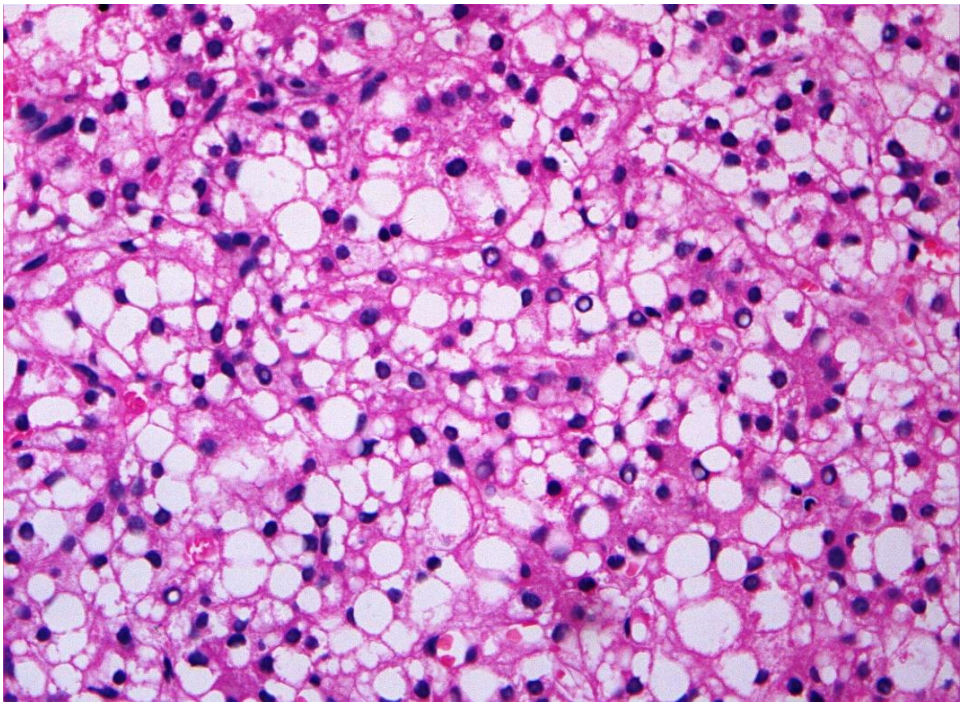


Figure 16: HB with extensive predominantly macrovesicular steatosis, H&E **400X**

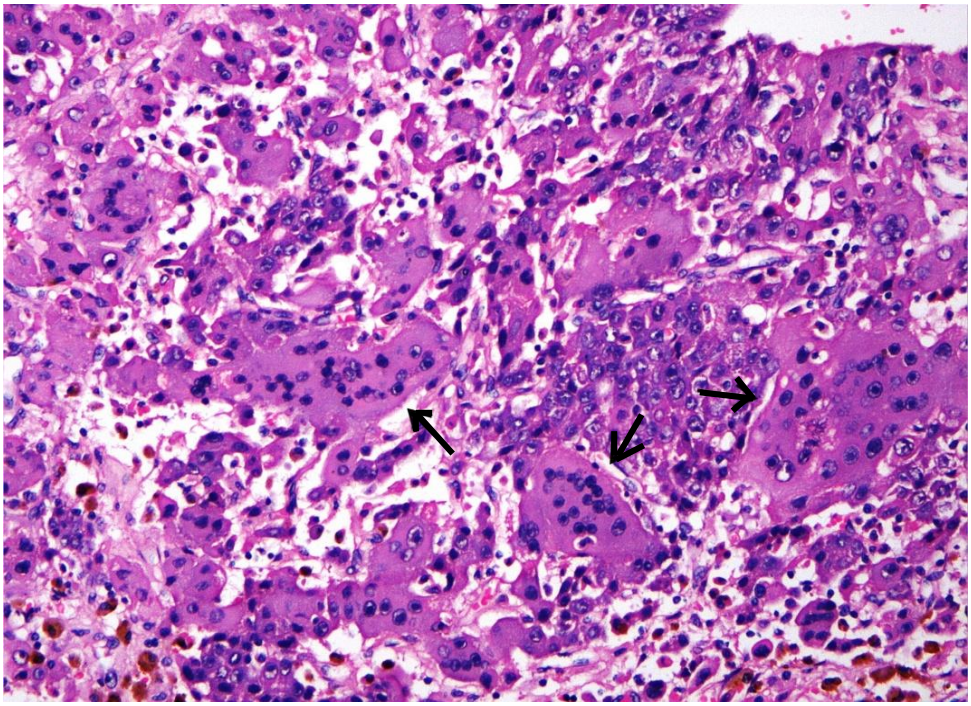


Figure 17: HB with multinucleation/ tumour giant cells (arrows), H&E **200X**

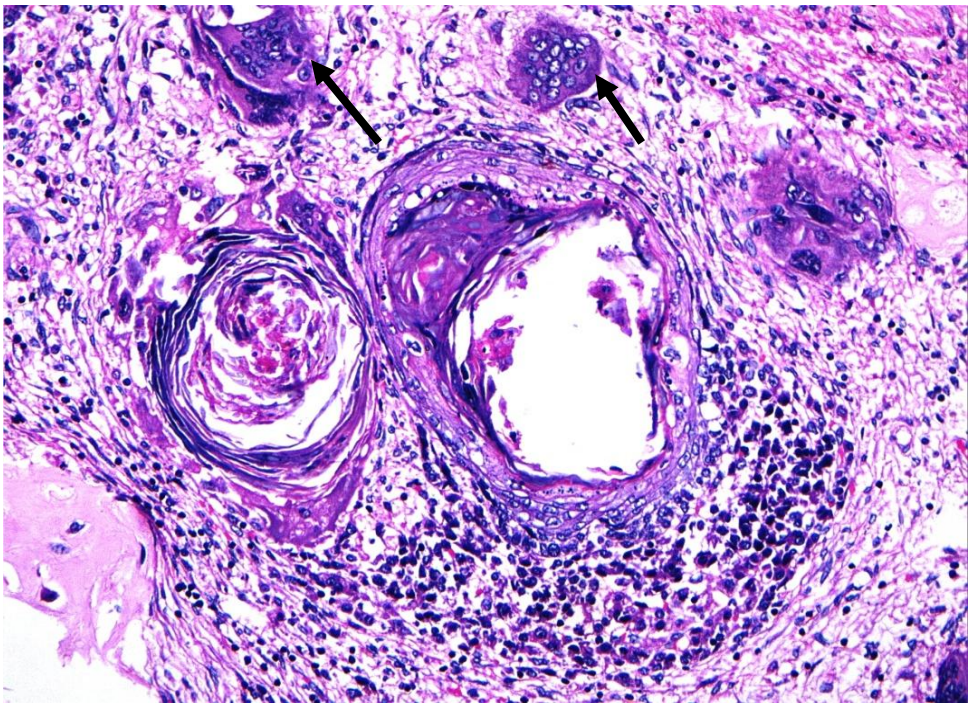


Figure 18: HB (post chemo) with squamous differentiation and giant cell reaction (arrows), H&E **200X**

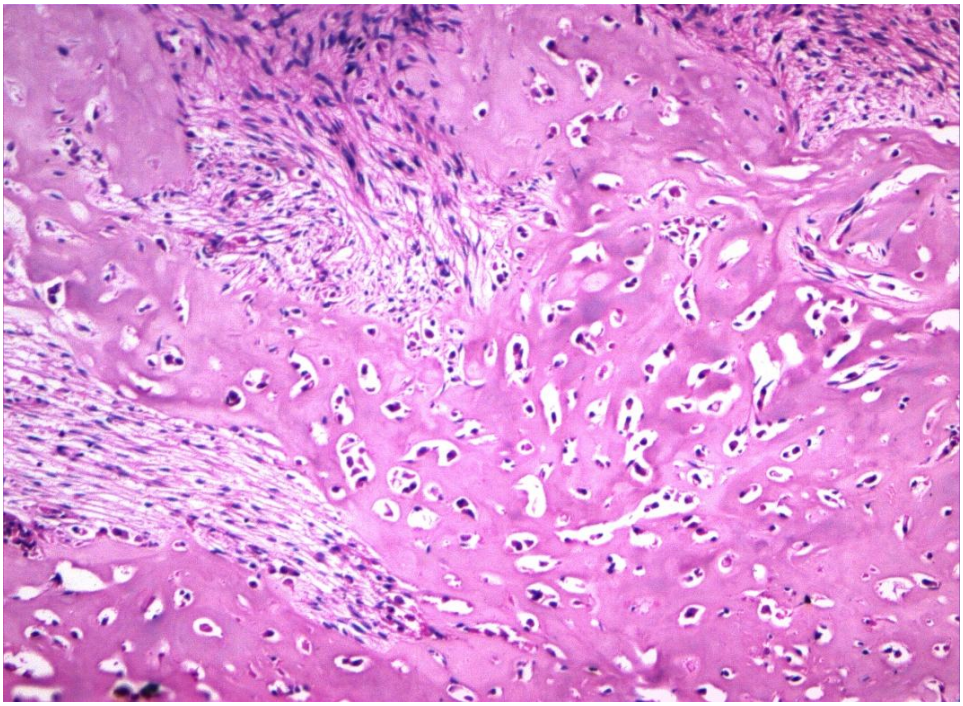


Figure 19: HB with extensive ossification (post-chemo), H&E **100X**

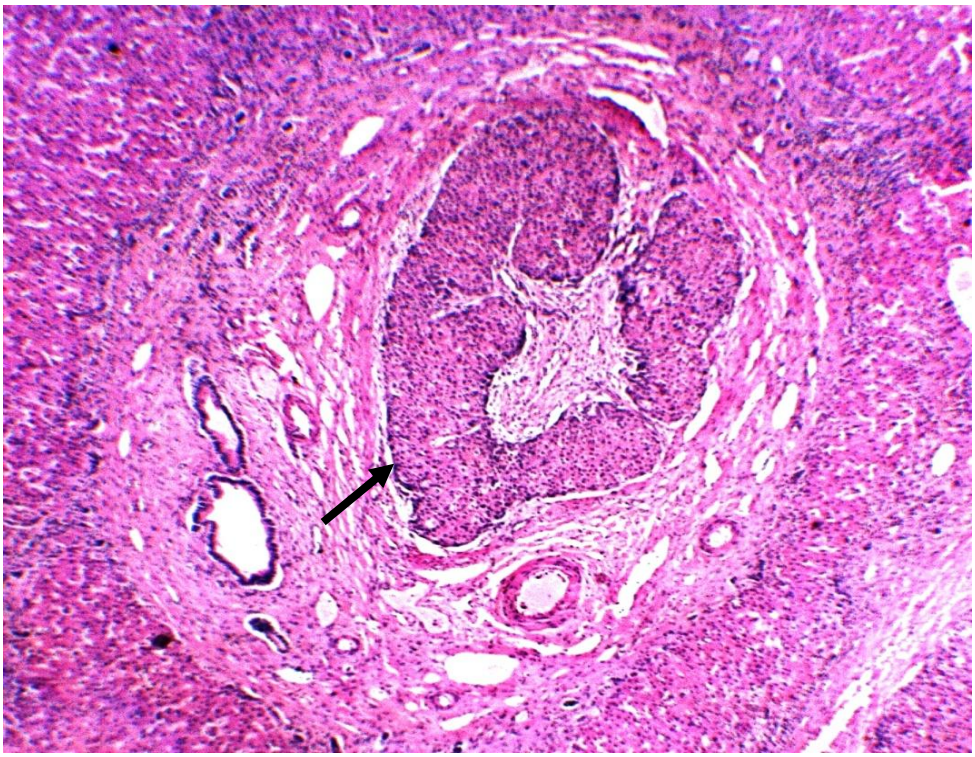


Figure 20: HB – Tumour embolus within a portal vein radicle (arrow), H&E **40X**

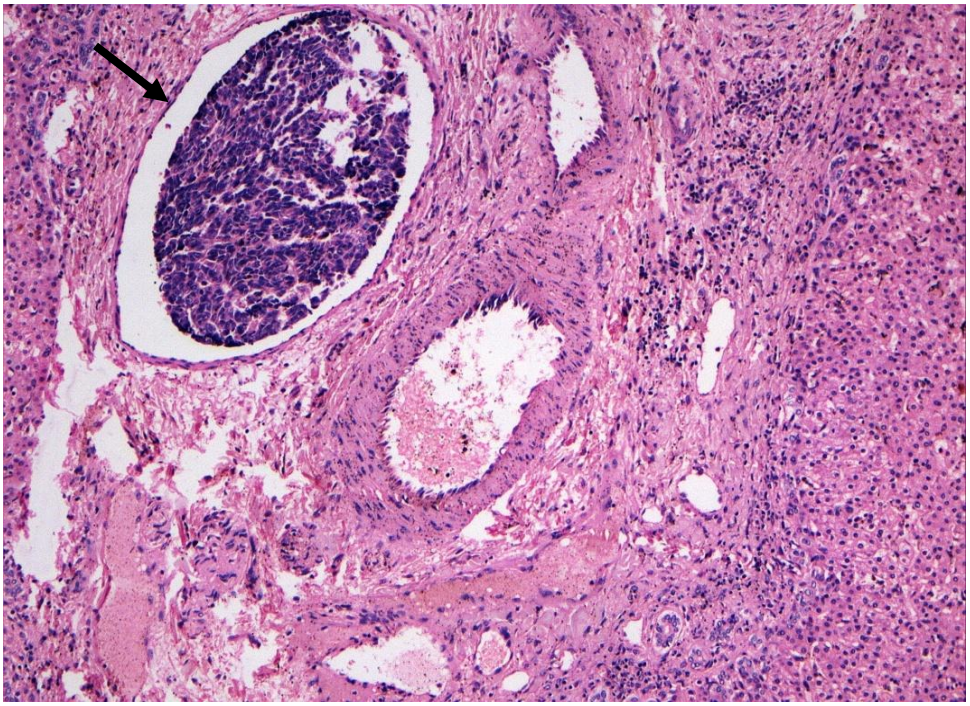


Figure 21: HB – Tumour embolus within a portal venule in another case (arrow), H&E **200X**

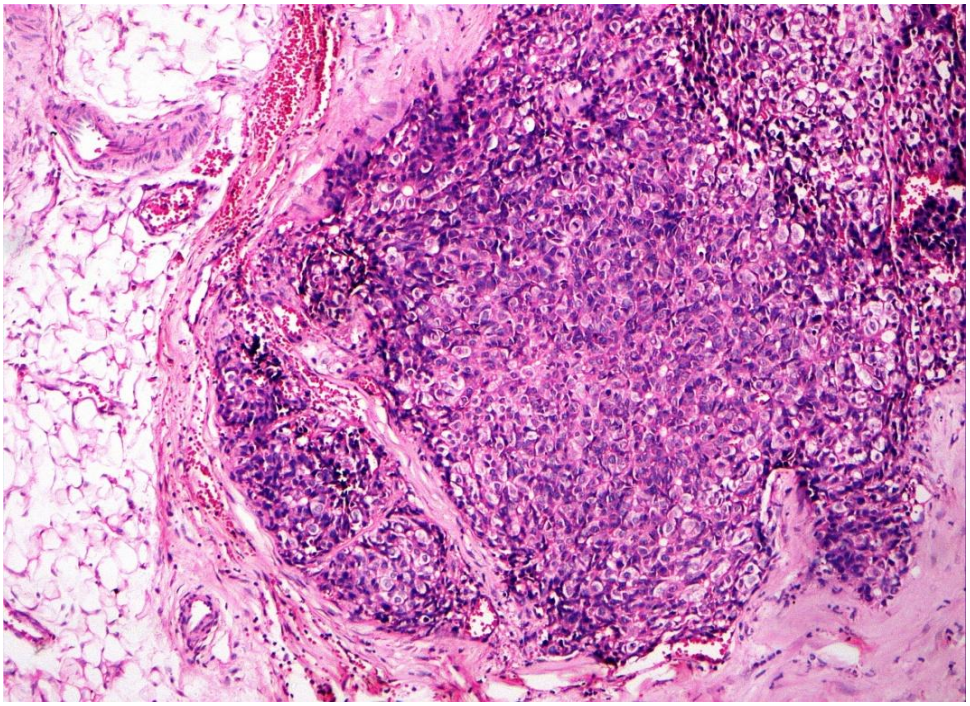


Figure 22: Metastatic HB in omentum, H&E **100X**

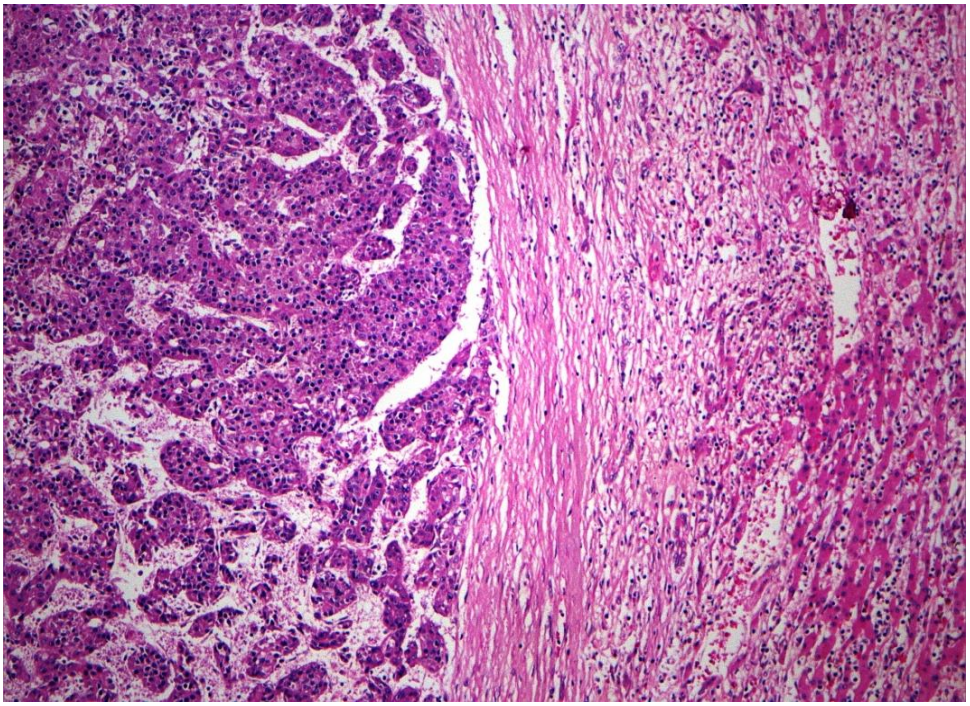


Figure 23: Recurrent HB, H&E **100X**

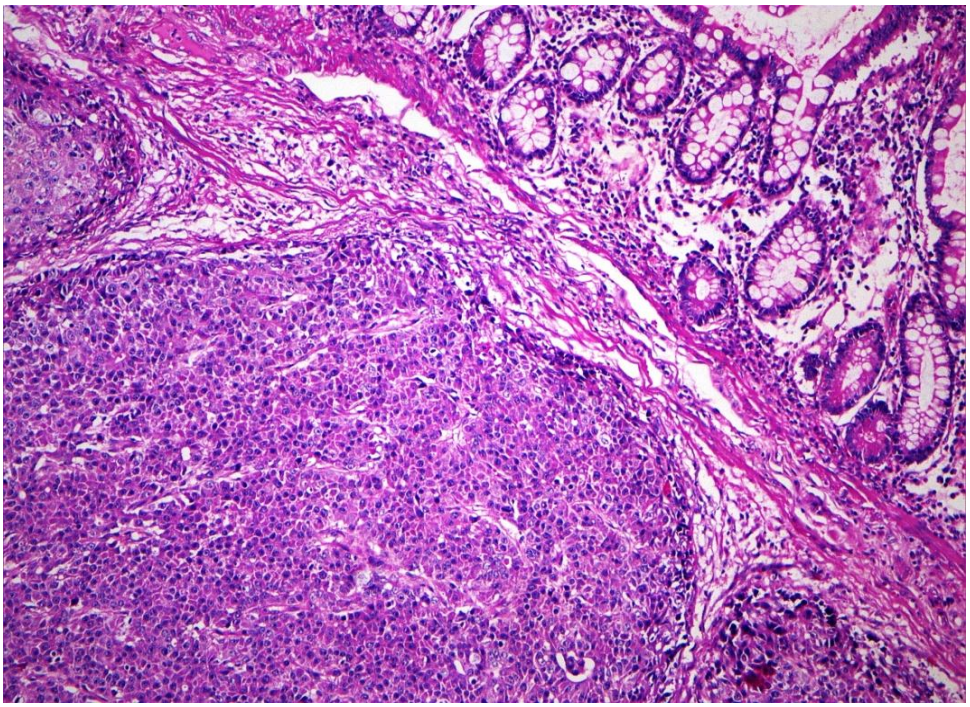


Figure 24: Metastatic HB in ileum, same case as above, H&E **100X**

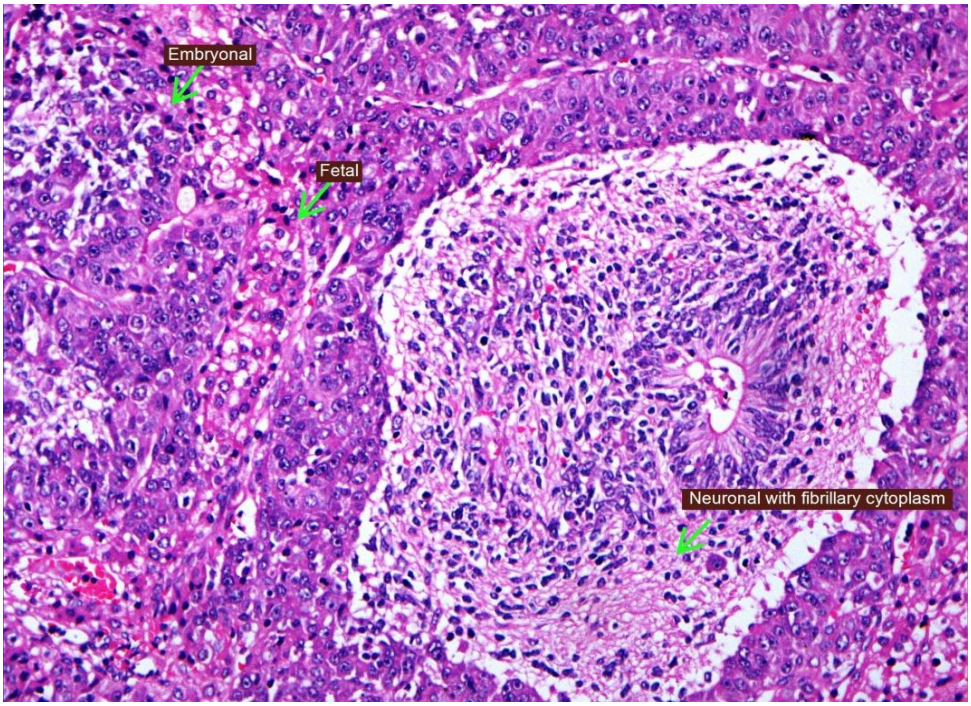


Figure 25: Metastatic HB in lung – Teratoid subtype, H&E **200X**

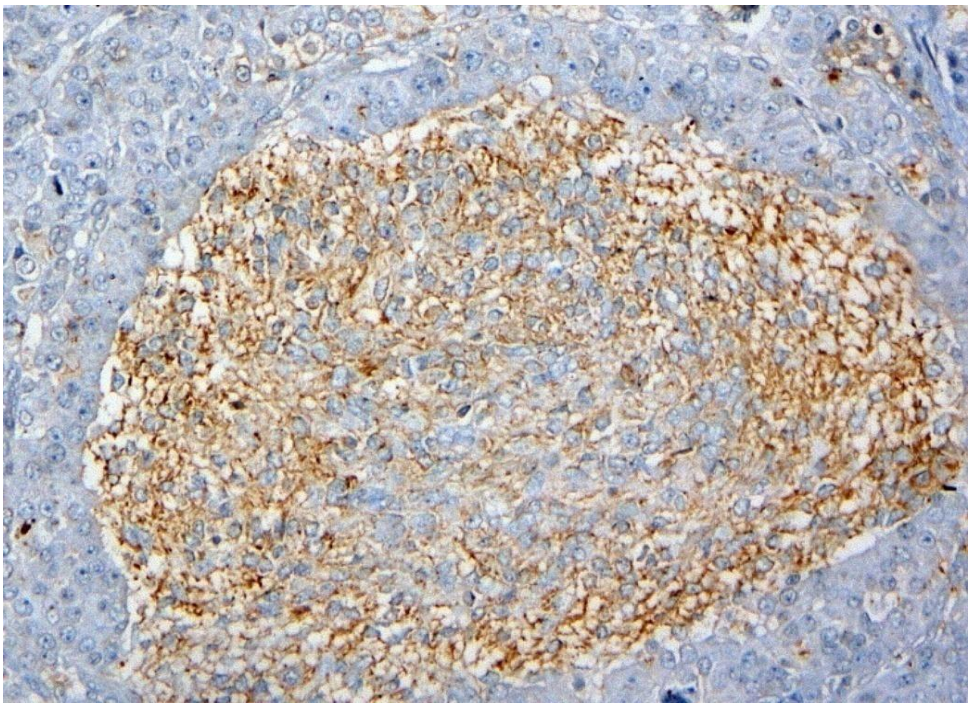


Figure 26: Metatstatic HB in lung – Teratoid subtype, neuronal component staining for synaptophysin, same case as above, IHC **200X**

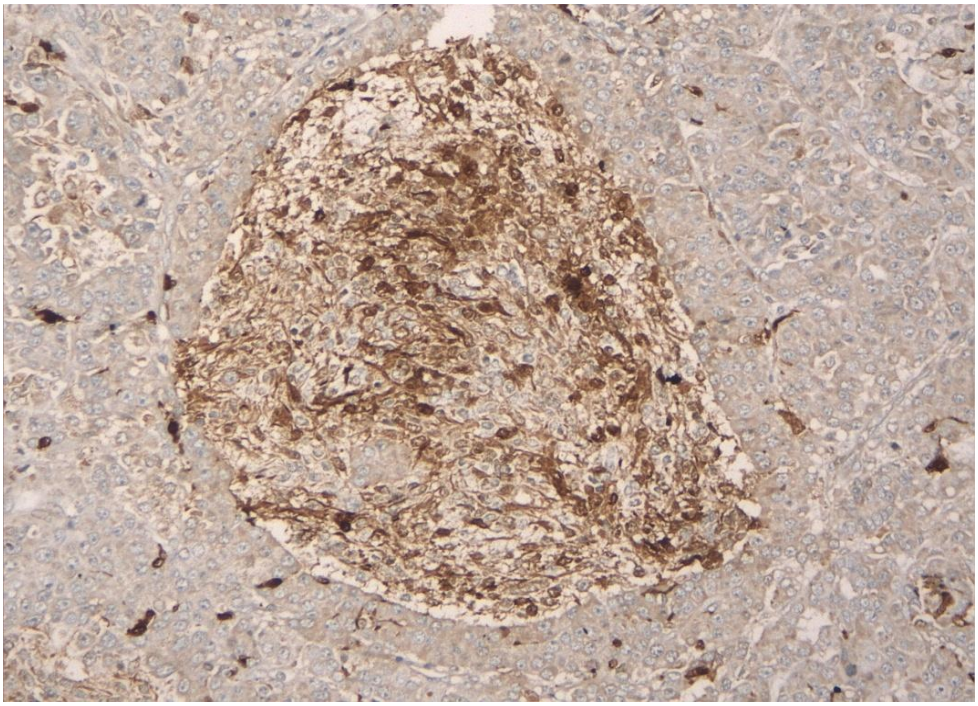


Figure 27: Metatstatic HB in lung – same case, staining positive for S100, IHC **200X**

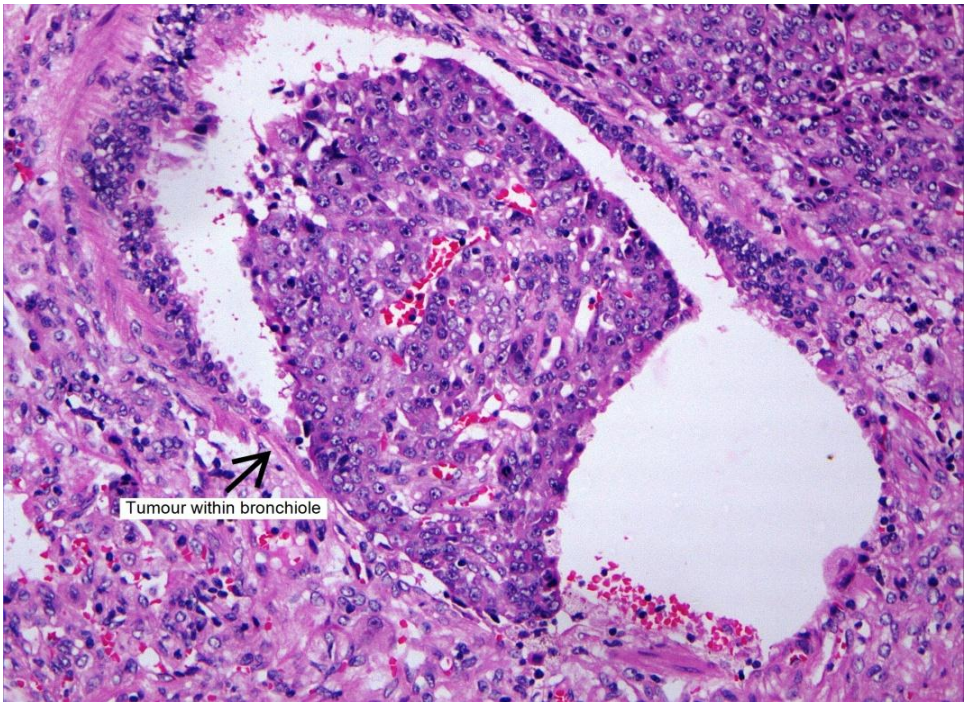


Figure 28: Metastatic HB in lung - Invasion into bronchiole, same case as above, H&E **200X**

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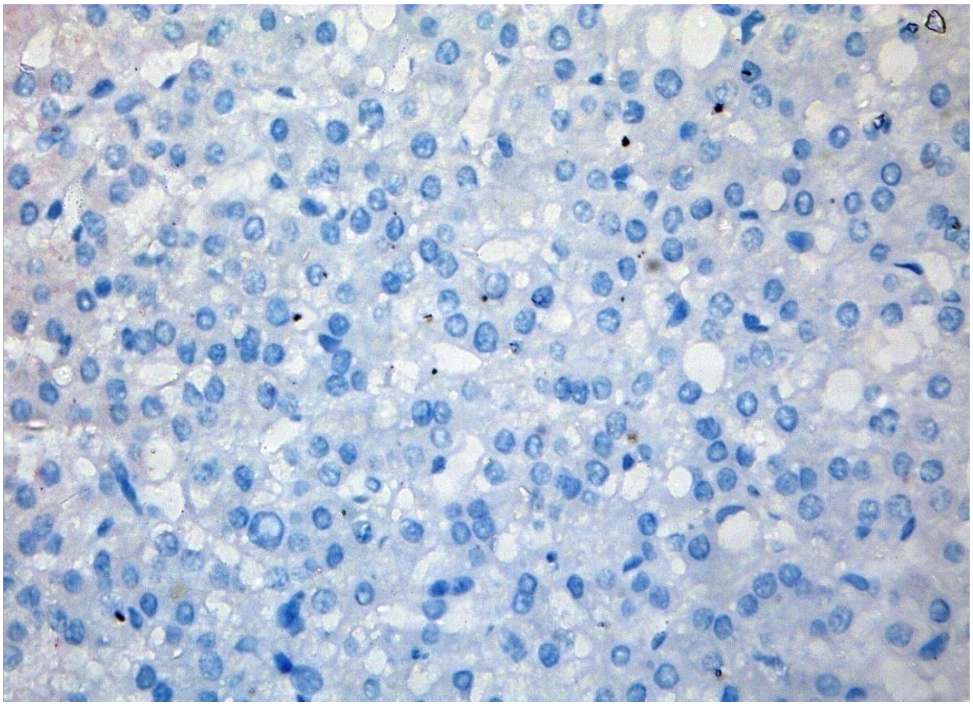

Figure 1: HB, pure fetal subtype – No staining for CK19, IHC **40X**

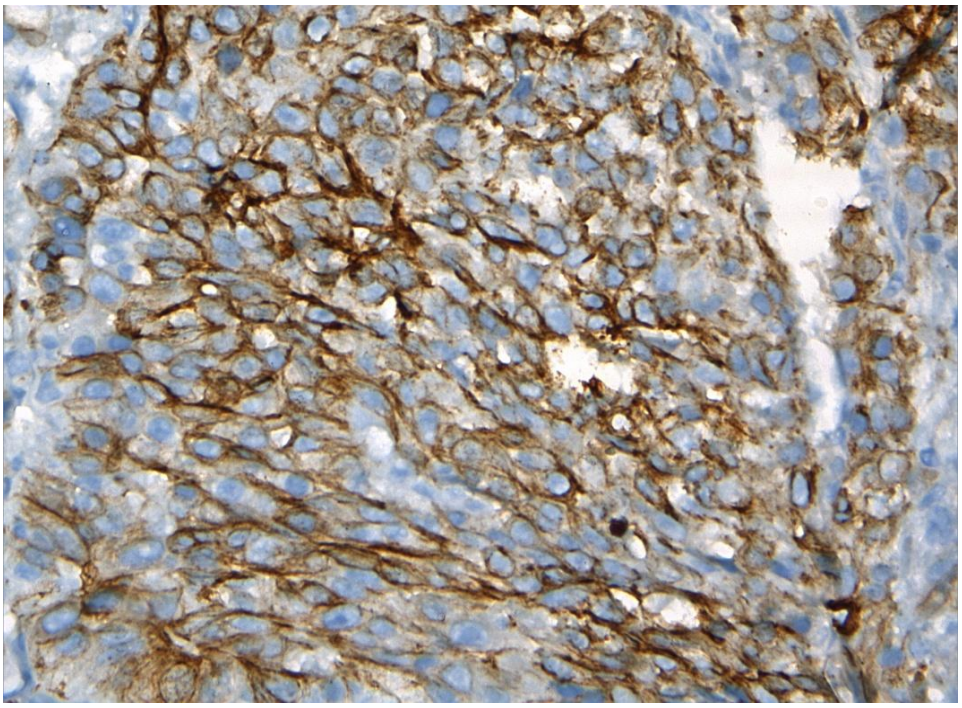

Figure 2: HB, embryonal component – Moderate to strong staining for CK19, IHC **400X**

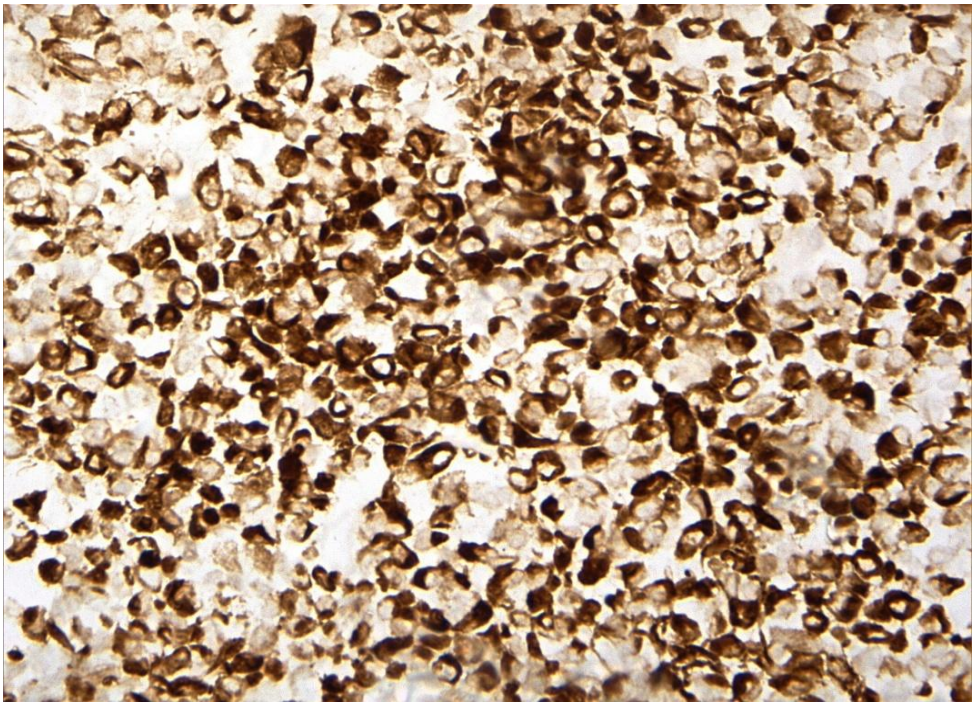

Figure 3: HB, SCUD subtype – Strong membranous staining for CK19, IHC **400X**

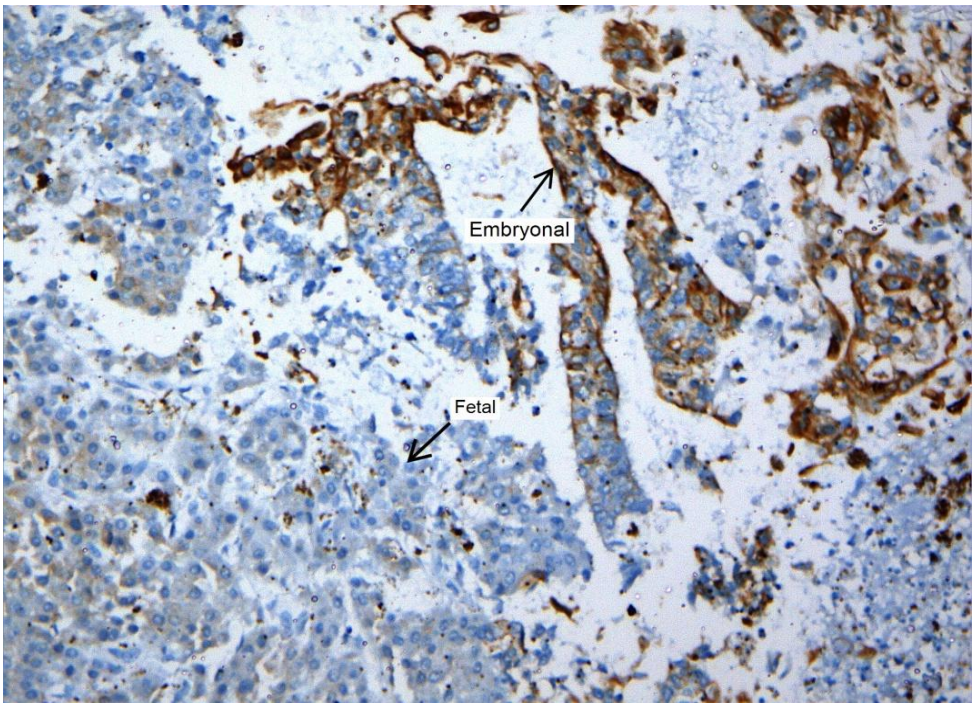

Figure 4: HB – Positive staining for CK19 in the embryonal component and negative staining in the fetal component, IHC **200X**

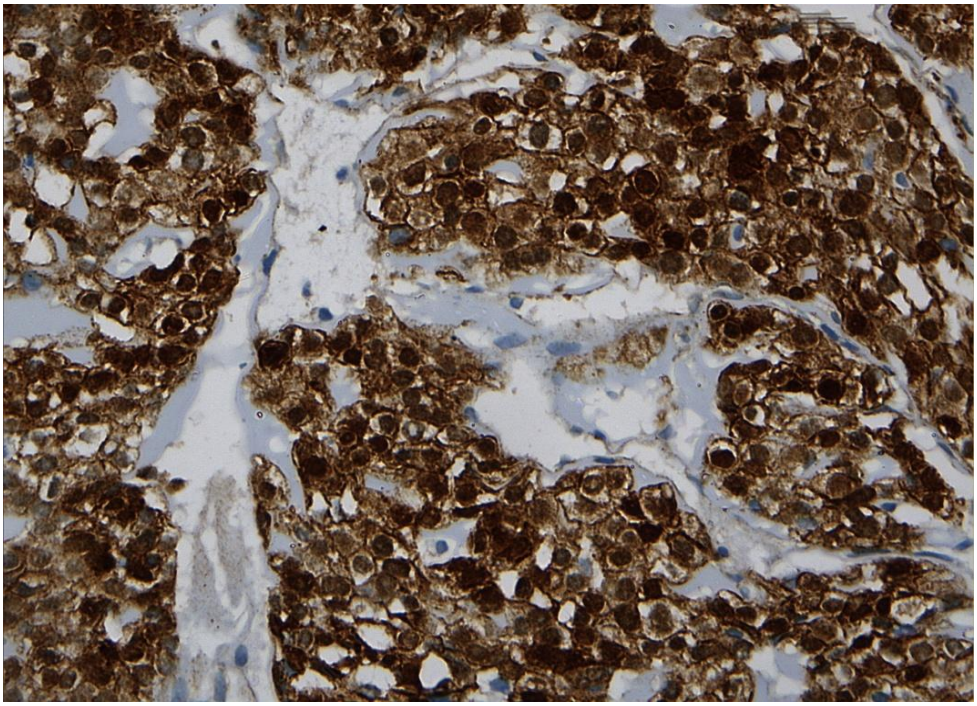

Figure 5: HB, fetal subtype – Strong nuclear and cytoplasmic staining for beta-catenin, IHC **400X**

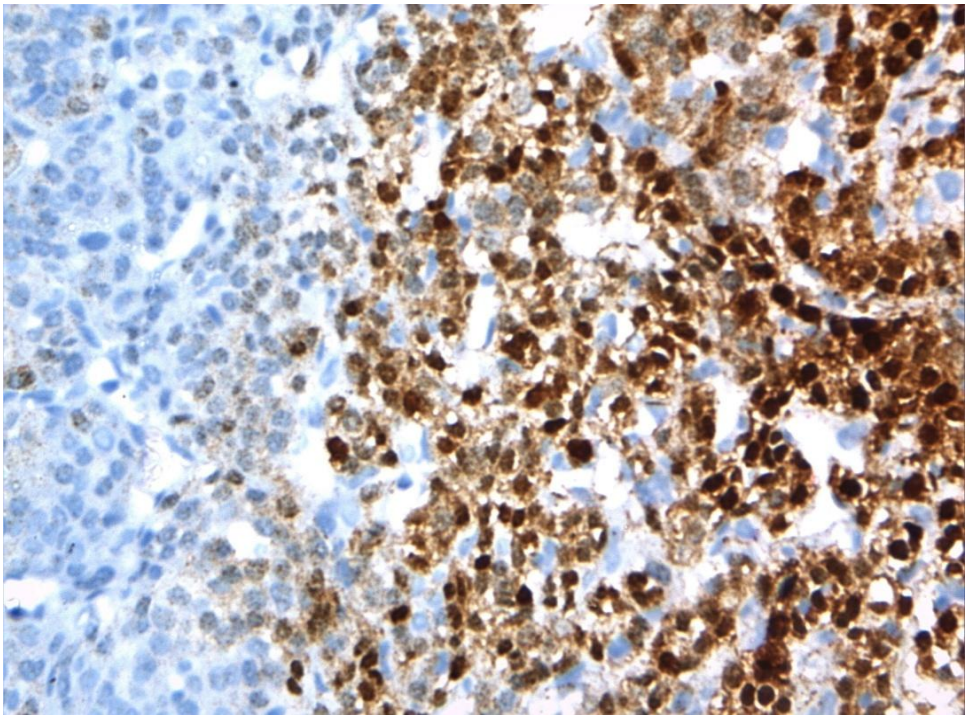

Figure 6: HB, embryonal component – Strong nuclear and cytoplasmic staining for beta-catenin, IHC **400X**

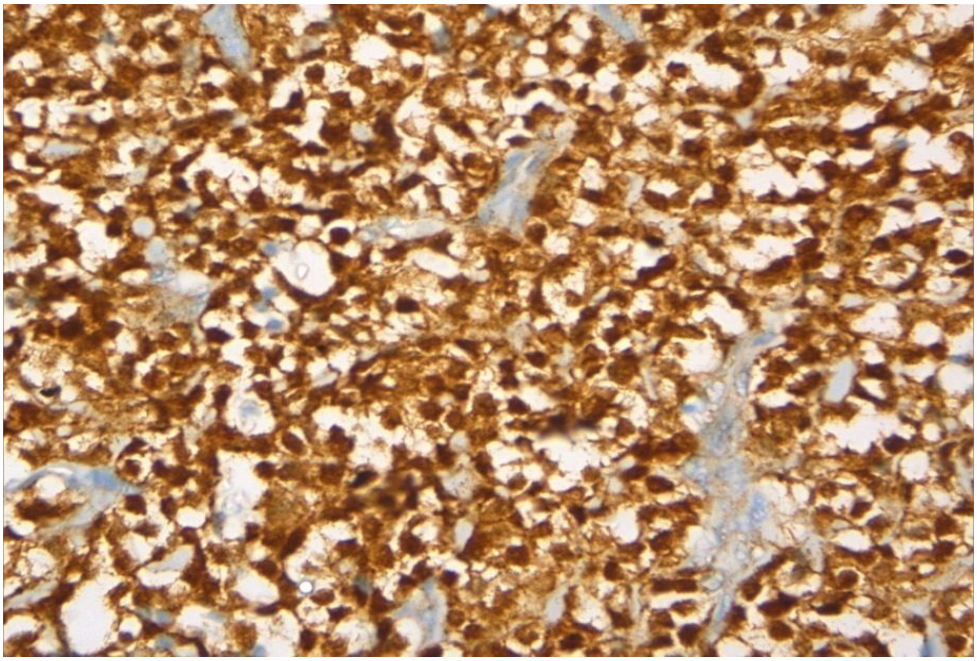

Figure 7: HB, SCUD subtype – Strong nuclear and cytoplasmic staining for beta-catenin, IHC **400X**

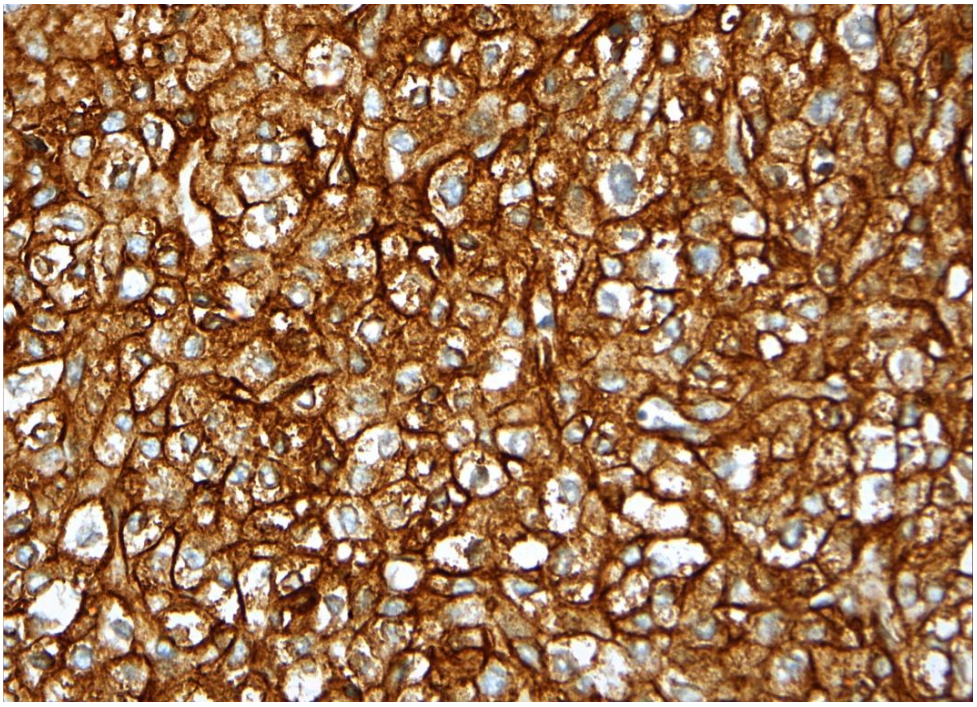

Figure 8: HB, fetal subtype – Strong diffuse membranous staining for EpCAM, IHC **400X**

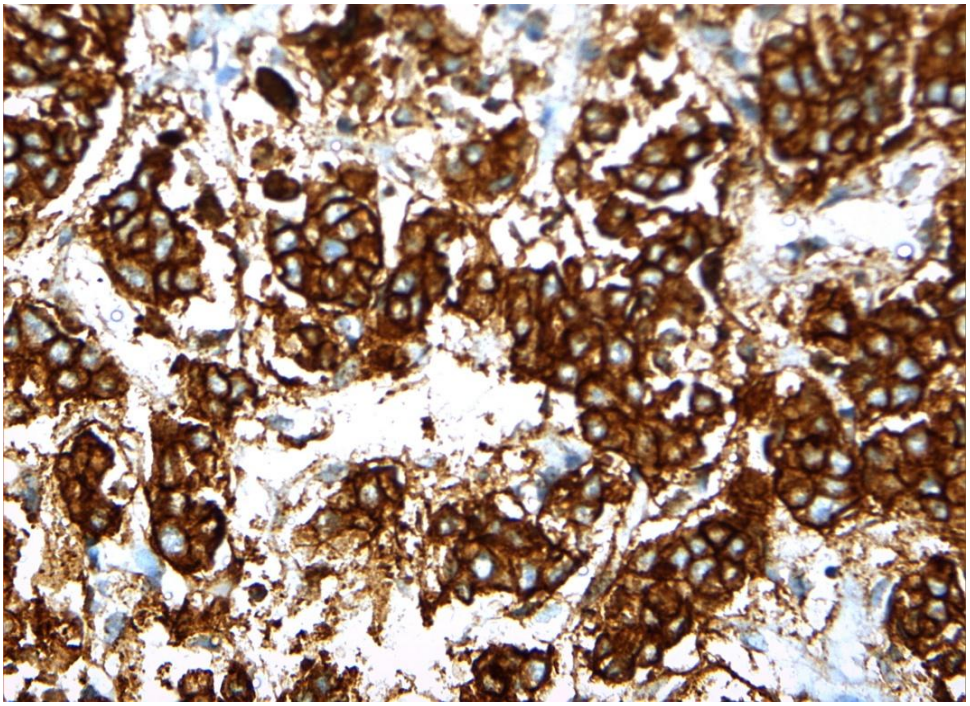

Figure 9: HB, embryonal component – Strong membranous expression for EpCAM, IHC **400X**

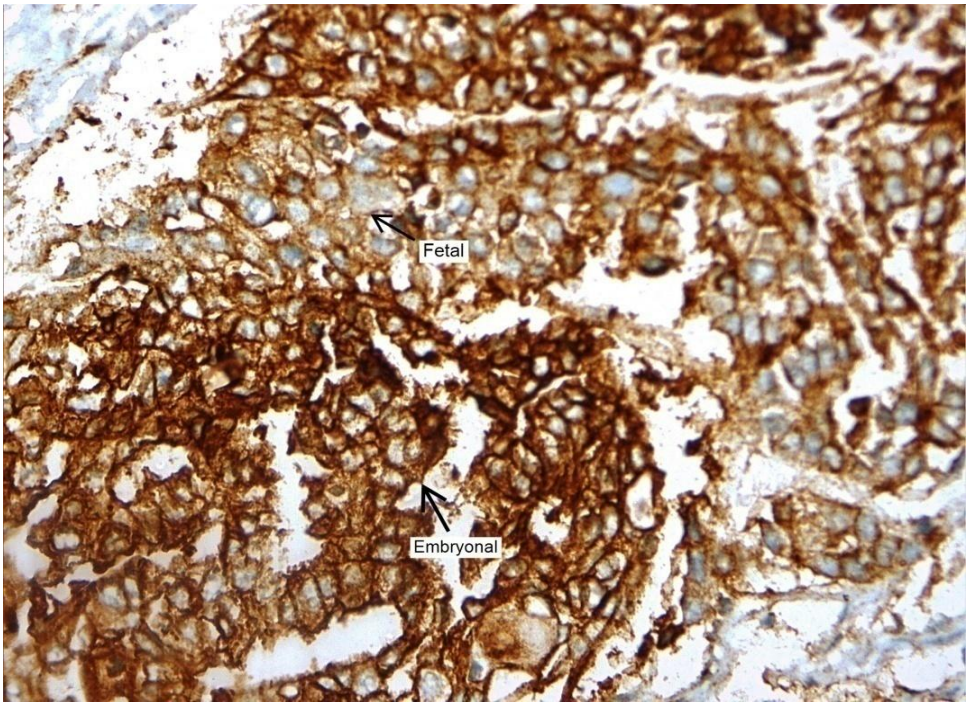

Figure 10: HB - EpCAM staining in fetal and embryonal (stronger) components, IHC **400X**

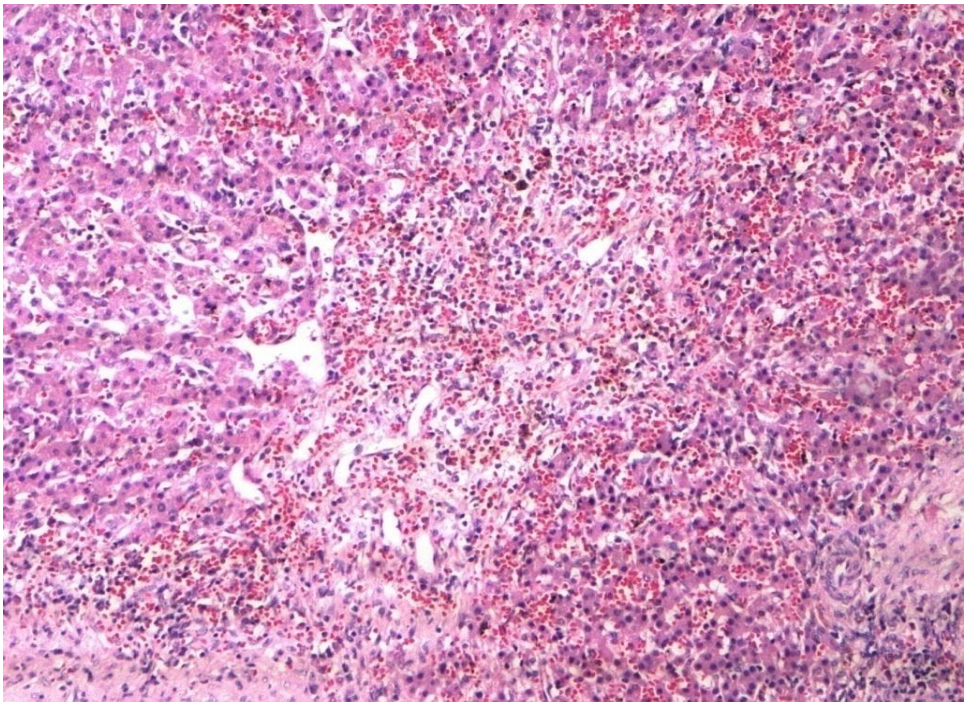

Figure 11: HB with maturation (post-chemo), H&E **100X**

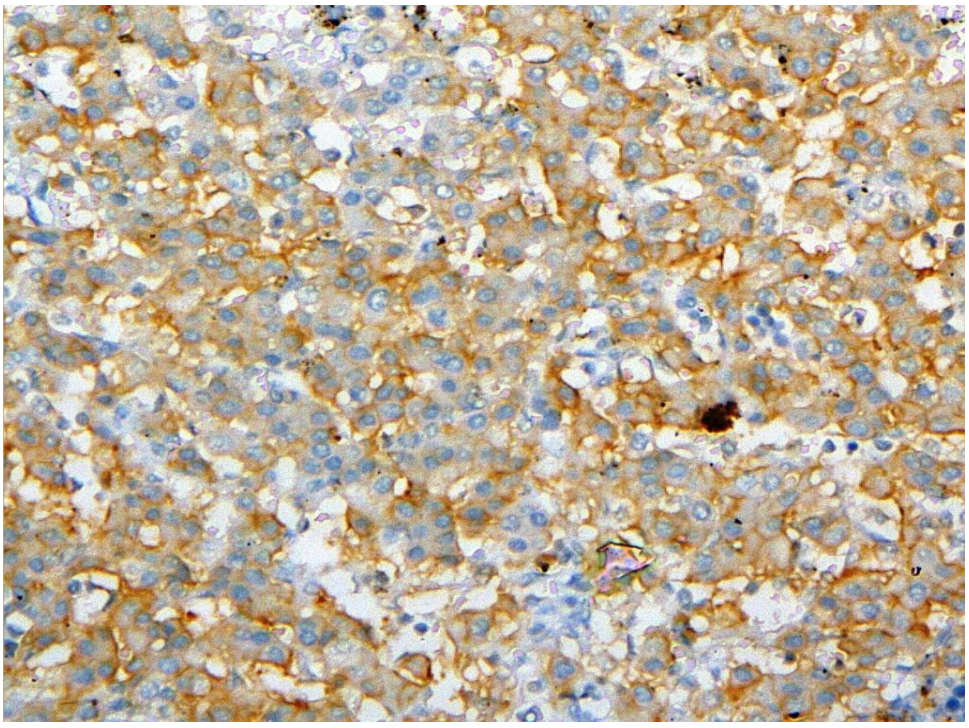

Figure 12: HB with maturation - EpCAM highlighting the mature tumour cells, same case as above, IHC **200X**

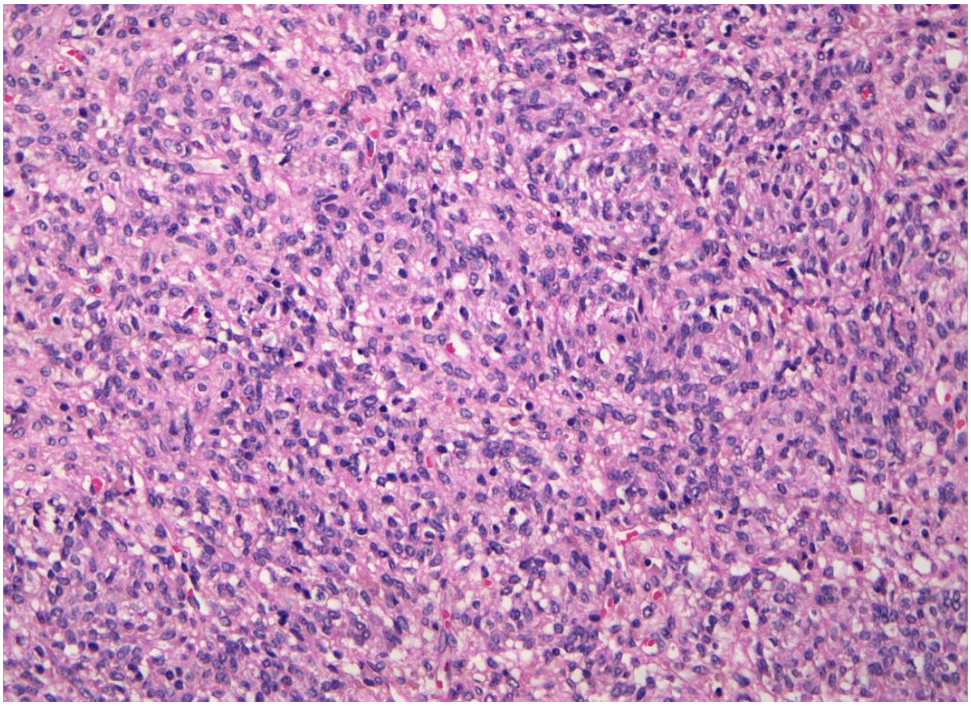

Figure 13: HB – Whorls of mesenchymal cells (post-chemo), H&E **200X**

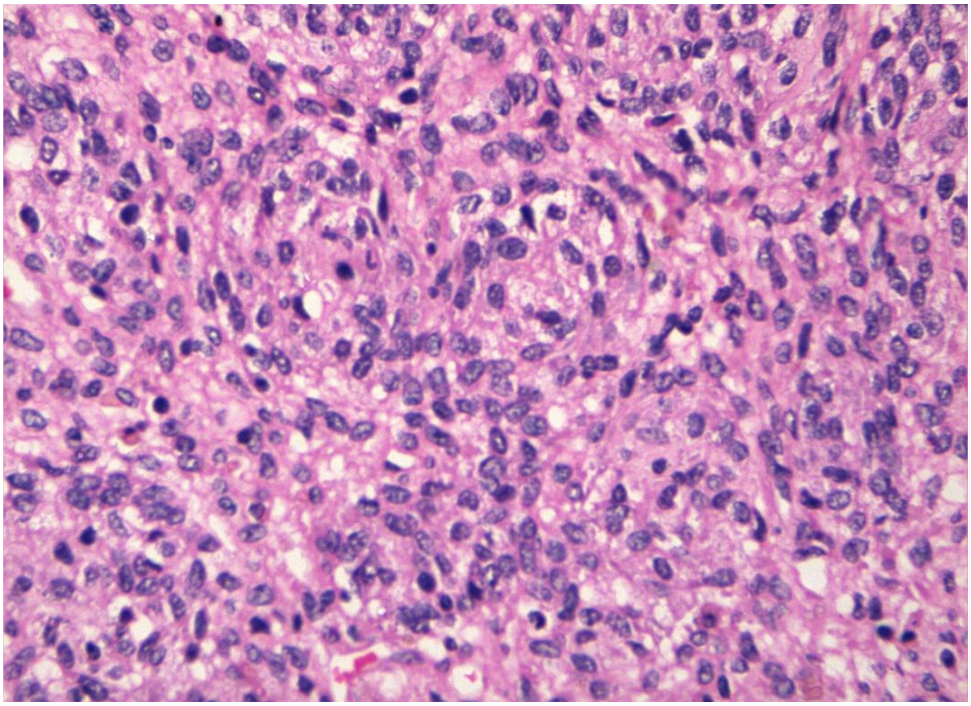

Figure 14: HB – Whorls of mesenchymal cells (post-chemo), high power, H&E **400X**

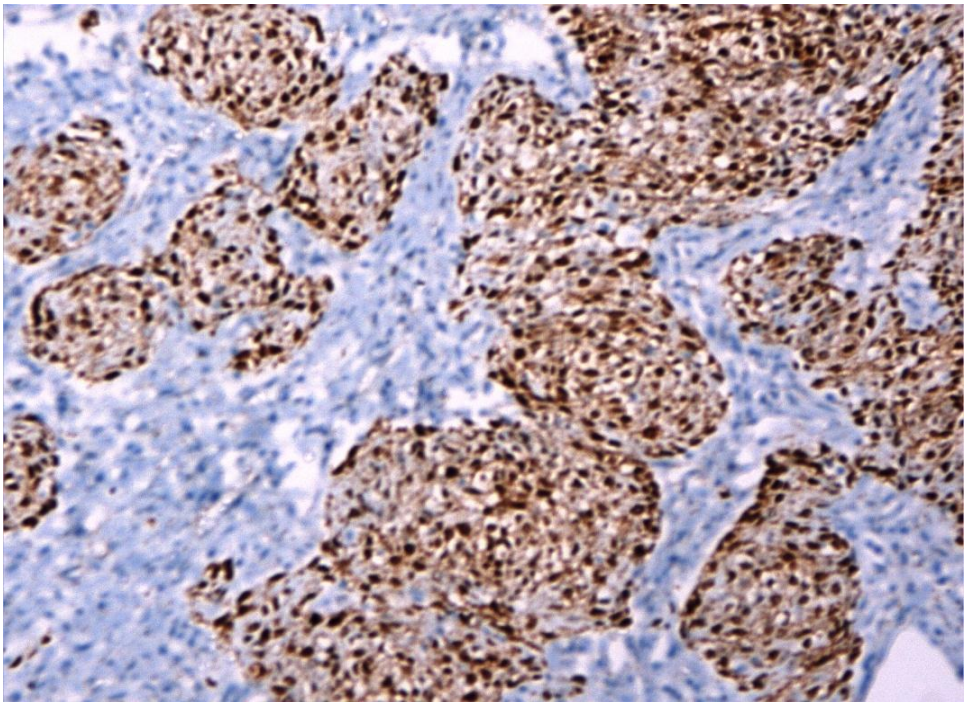

Figure 15: HB - Whorls of mesenchymal cells staining (nuclear) for beta-catenin, same case, IHC **200X**

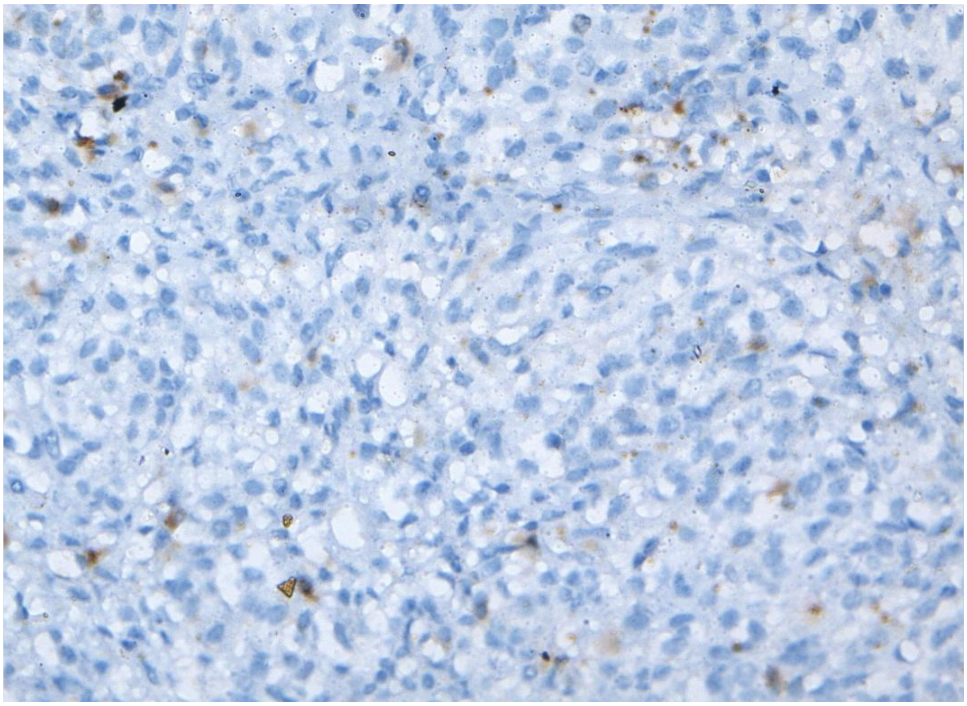

Figure 16: HB - Whorls of mesenchymal cells negative for pan cytokeratin, same case as above, IHC **400X**

DISCUSSION

DISCUSSION

This study included 55 cases of HB with 22 biopsies, 11 resections and 22 cases with both biopsy and resection. Three patients who were treated surgically for the primary tumour underwent resections of recurrent tumour and two of these patients also underwent resection of the metastatic tumour in ileum and omentum. One other patient also underwent resection of the metastatic tumour in the lung.

The mean age of the patients at presentation in this study was 2.3 years and 78% of our patients presented within two years of life. This is similar to the study by Schmidt *et al*, in which two-thirds of HB occurred in patients within first two years of age and >90% in less than 5 years of age(12,14). There was only one patient in our study who presented at 13years of age.

HB occurs more commonly in boys than in girls (14). However, there is a variation in the male to female ratio ranging from 1.2:1 as in the Surveillance Epidemiology and End Results Program (SEER) of the United States in the year 1975 – 1995(72)to 2.9:1 in the Taiwan population(73). However, Sarangarajan *et al* have found slightly higher incidence among the girls, with a male to female ratio of 1:1.4 (47). In our study, the boys outnumbered the girls with a male to female ratio of 2.1:1. The reason for the gender distribution of HB is not clearly understood.

Clinically, HB presents as an asymptomatic abdominal mass. The most common presentation in this study was presence of an abdominal mass accounting for 38.2% , which is similar to the results in other studies(47,74). The other common symptoms in our patients were fever and abdominal pain. Jaundice as a presenting symptom was

seen in 4 cases (7.27%), which is slightly higher than in the literature, where it is reported as <5% (14).

Antenatal presentation of HB is very rare (74) and congenital HB were found to have a distinct biologic behaviour when compared to those diagnosed in neonatal period including a higher frequency of pure fetal subtype, systemic metastasis and poor outcome (75). One patient in our study was diagnosed with a liver mass in utero, during routine antenatal ultrasound and was diagnosed to have a pure fetal HB. However, the follow up details of this patient was not available.

Though an increased risk of HB is associated with Beckwith Wiedemann syndrome, hemi hypertrophy and familial adenomatous polyposis, none of our patients had these associations(13). The common associations seen in our patients were neuroblastoma in 2 patients (3.63%),horse-shoe kidney in 2 patients (3.63%) and medullary sponge kidney, gonadal enlargement with precocious puberty and gastroschisis in one each. Most of these associations have not been reported previously.

One patient with gonadal enlargement and precocious puberty had elevated levels of beta-hCG(167IU/ml) at presentation. The association between HB and beta-hCG has been reported previously and whether the tumour cells secrete testosterone or whether they stimulate the Leydig cells of testis is not clear (76). It has also been found that hepatoblastoma associated with beta-hCG, called virilising hepatoblastoma are associated with a grim prognosis (77). Two of three (66.67%) patients with elevated beta-hCG in our study had metastasis to the bones and lung respectively at presentation and died of disease, 6 months after initial diagnosis.

Elevation of beta-hCG has also been associated with the presence of multinucleate tumour cells, as described by Watanabe *et al* in the Japanese population (78). However, this association was not seen in our study.

Serum AFP levels at the time of initial diagnosis was available in 46 patients, of which 41 (97.6%) showed abnormally high levels with a median value of 300000IU/mlwhich is similar to the results observed by Tsuchida *et al* (79). At diagnosis, only one patient had normal serum AFP level (4.32IU/ml) and was diagnosed to have SCUD subtype of HB and died of disease, 7 months after the initial diagnosis. This is in concordance with the previous studies, that low AFP levels (<100IU/ml) are associated with SCUD subtype and an adverse outcome(13,80,81). Decrease in the serum AFP levels following chemotherapy and surgery was noted in 22/28 (78.57%) and the difference between the groups was statistically significant.

It is well known that patients with no fall or persistent increase in AFP following chemotherapy have a residual tumour or recurrence or a metastatic lesion (21,82). Six patients in our study had persistent increase in AFP, of which two patients had multiple metastatic nodules in the lung, two had a recurrent tumour with metastasis at a distant site and one had chemo resistant HB with no decrease in tumour dimension.In our study, one patient with persistent increase in AFP had MEM with teratoid subtype of HB and the association between the two has not been documented before.

More than 50 % of the patients in our study belonged to PRETEXT II, 13/25 (52%) and 6/25 (24%) were in PRETEXT III. When the PRETEXT score was compared with

death, it was found that a significant number of patients in PRETEXT III and PRETEXT IV (83.33% and 100%) succumbed to the disease when compared to those in PRETEXT I and PRETEXT II (66.66% and 7.69%) . In a study conducted by Meyers *et al* in California, the 5 year overall survival rate for patients in PRETEXT I was 88.9% when compared to only 30.9% for PRETEXT IV(80). Similar results have also been published by Hor *et al* where survival of patients in PRETEXT II was better when compared to those in PRETEXT III and IV (83). Though not statistically significant our study also showed better event free survival (EFS) of 63.89 months for patients in PRETEXT I & II,when compared to 43.77 months for patients in PRETEXT III and IV.

Tumour characteristics:

The tumour was located more commonly in the right lobe of liver (54.55%), when compared to 20% in the left lobe and 25.45% of tumours involved both the lobes. This is similar to other studieswhere the right lobe involvement was found to be three times more common than the left(13) and 20-30% of tumours involved both lobes(31,84). Around 81.08% of patients in our study had a single tumour nodule and the remaining had multiple tumour nodules, which was similar to the study by Gupta *et al* in which 20.83% tumours were multifocal (35). Multifocality has been proved to be an important prognostic factor with lower EFS rates, reported by Saettni *et al* (85). In our study, the mean EFS rate of patients with a multifocal tumour was only 8.20months when compared to 58.77months for those with a single tumour and this was statistically significant.

The mean gross dimension of the tumour by radiology was 10.06cm (3.5-18cm) and following chemotherapy was 6.70cm (1.5-15cm), similar to the study by Gupta *et al* (35). There was a statistically significant difference between the two groups, indicating response to chemotherapy and this was not compared in the previous studies on HB.

On gross evaluation of the resected tumours, most of the tumours (84.15%) had a solid cut surface and 15.15% had both solid and cystic appearance. However, the gross appearance of the tumour varies based on the effect of chemotherapy and the tumours can have a variegated cut surface with areas of haemorrhage, necrosis, hyalinisation and ossification(31,35). In our study, two tumours had extensive ossification and had a hard grey white appearance grossly.

Tumour characteristics – Histological features:

The predominant histologic pattern in our study was trabeculae, identified in 43 of 55 (78.18%) of tumours, followed by cords and solid nests in 41.81% (23 tumours). The most common epithelial subtype in the pre-chemotherapy group was the predominantly fetal subtype in 19 of 44 (43.18%) cases followed by the mixed epithelial subtype in 18/44 (40.91%) cases. This is similar to the results published by Purcell *et al* in the study of patients enrolled in the SIOPEL 3 trial(86), where fetal and mixed epithelial subtypes were seen in 42.50% and 46.81% respectively. In the post-chemotherapy group mixed epithelial subtype was the commonest in our study, accounting for 54.83% of cases. This is in contrast to the study by Gupta *et al* from North India, in which the most common subtype was fetal (62.5%) in the post-

chemotherapy group (35). In our study, SCUD was found in one case, accounting for 3.2%, similar to that reported in the literature (31). Two tumours in this study had MEM with teratoid features.

Extramedullary haematopoiesis was noted in 47.72% and 72.23% of tumours in the pre and post-chemotherapy groups, with the erythroid cell line being the most common. This is similar to the studies by Gupta *et al* (35) and von Schweinitz *et al* (87) where EMH was present in 54.17% and 66.67% of tumours respectively. Megakaryocytes were present in 6.82% and 18.18% of tumours in both the groups respectively. EMH was noted in the fetal component of the tumour and this correlates with the fact that liver is the major site of haematopoiesis in the fetal liver. It has been described that at certain stages of differentiation, the tumour cells induce the pluripotent stem cells to differentiate along the erythroid cell lines. The tumour micro-environment, the humoral factors and cytokines are important factors that induce haematopoiesis in HB (88).

The average number of mitosis/10hpf was 2 and 1 in fetal and 6 and 11, in embryonal components in the pre and post -chemotherapy groups respectively. When a cut off of 5/10 hpf was used to stratify the tumours, in the pre-chemotherapy group, 85.3% and 36% of tumors with fetal and embryonal components had a mitotic count of ≤5/10hpf, respectively. Following chemotherapy, all (100%) fetal had a mitotic count of ≤5/10hpf when compared to embryonal component (47%), which was statistically significant.

This finding is similar to the study by Sarangarajan *et al* where the tumours were evaluated with proliferation marker PNCA (proliferating cell nuclear antigen) and it was found that the embryonal component showed no decrease in mitosis, while the fetal subtype displayed substantial decrease in mitosis following chemotherapy (47).

The last patient in thisstudy (pre chemotherapy group) with fetal subtype had a mitotic count of 15/10hpf and was diagnosed as mitotically active fetal subtype. This patient was in PRETEXT IIIwith inoperable tumour and is on palliative treatment.

Bile production is commonly seen in HCC and is very rare in HB (31). In our study, cholestasis was noted in only 9.09% in both pre and post-chemotherapy groups. Steatosis was seen in 31.82% and 21.21% of tumours in the pre and post chemotherapy groups respectively, which is similar to the study by Gupta *et al*, where it was seen in 16.67% of tumours, following chemotherapy (35). It is known that the fetal subtype of HB may undergo extensive steatosis following chemotherapy (89). In our study, fetal subtype was commonly associated with steatosis when compared to embryonal and SCUD subtypes in the pre-chemotherapy group, which was statistically significant. However, this could not be compared in the post-chemotherapy group because of the small sample size. To our knowledge, there are no previous studies that have correlated fatty change with the epithelial subtype in HB, in the pre-chemotherapy biopsies.

The mesenchymal elements seen in our study were osteoid in 18.18% and 75.76% and fibrous stroma in 4.54% and 36.36% of tumours in the pre and post chemotherapy groups respectively. The higher percentage of osteoid noted in the post-chemotherapy

group is similar to the study by Saxena *et al*, where osteoid was seen only in 36% of pre-chemotherapy samples and was found to occupy <5% surface area, while it was seen in 82% of post-chemotherapy specimens occupying >40% surface area(34). Increase in the osteoid following chemotherapy could represent maturation of the epithelial elements following chemotherapy. Furthermore, a direct correlation between the prognosis and proportion of osteoid has been proved in the study by Heifetz *et al* (90). The two patients, who had extensive ossification in this study, had no evidence of recurrence or metastasis at two years of follow up.

Von Schweinitz *et al*(91)have described the prognostic significance of vascular invasion (radiological and microscopic) in HB, and found that children with vascular invasion had a lesser disease free survival (55.2%) than those without (88.1%). However, to our knowledge, there are no studies that have described microvascular invasion in the tumour and the association with prognosis. Micro vascular invasion was noted in 14 of 33 (42%) tumours in our study. Though not statistically significant, the patients without MVI had EFS of 72.83 months when compared to 46.23 months in those with MVI.

The various chemotherapy induced changes noticed in our study were hyalinisation (78.78%), ossification (75.76%), recent haemorrhage (69.70%), haemosiderophages(66.67%), necrosis (63.64%), giant cell reaction (27.27%), squamous differentiation (21.21%) and calcification (48.48%). Other features noted include dense lymphoid aggregates within the tumour, myxoid change and multinucleation. Though these changes are described previously (35) , the exact percentage of these are not reported.

Another feature which was found in one case in our study was the presence of whorls and small clusters of spindle cells with bland nuclei surrounding the tumour cells. This was first described by Gupta *et al* as 'glomeruloid clusters' and was found to have strong nuclear expression of beta–catenin(35). In our study, these whorls/clusters stained strongly for beta-catenin, but did not express pancytokeratin. Thus in our opinion, these clusters could represent the stromal changes following chemotherapy and they may not be of epithelial origin.

Venkataramani *et al* (33), have analysed the percentage of necrosis and correlation with survival. In the present study we evaluated the percentage of viable tumour with survival, since this would assess the exact response to chemotherapy, by excluding other chemotherapy related changes such as hyalinisation, ossification etc in addition to necrosis. In this study, 55.58% of patients had ≥50% viable tumour. There was a statistically significant difference when patients with <50% (EFS: 109.90 months; no event) and ≥50% (EFS: 39.28 months) viable tumour were compared in terms of event free survival. When the percentage of viable tumour was compared with the histological subtype, it was found that 90.90% of tumours in MEM and the single SCUD subtype had >50% viable tumour. To our knowledge, this has not been reported previously.

In this study, tumours with a predominantly fetal subtype had a comparatively decreased chance of MVI, recurrence, metastasis and death when compared to other subtypes, though the difference was not statistically significant. Tumours with a pure fetal histology had a better outcome when compared to anaplastic or small cell histology as described in other studies(4,27,64). Haas *et al* have found a 97% survival

at 24 months for pure fetal histology when compared to only 52% for other subtypes (62).

In the current study, one case which had SCUD subtype had micro vascular invasion, metastasis and died due to disease. The other two cases with focal SCUD areas showed evidence of MVI and one patient had metastasis to the lung. This is also similar to the study by Hass *et al*, in which 16 children who had partial or predominant small cell morphology subtype of HB were studied, 10(63%) developed recurrence and 5 of these 10 patients died due to the disease (5). Our results correlate with the finding that SCUD pattern when present even as a minor component of the tumour, affects prognosis significantly. However Gupta *et al* (35)and Conran *et al* (56)have found no significant association between the histological subtype of the tumour and the survival outcome.

When the distance of the tumour from the resection margin was compared with death, it was found that 33.33% of cases with margin ≤0.5cm died of disease. In this study, it was also found that these patients also had other features of prognostic importance like MVI (4 cases), SCUD (1 case) subtype and lung metastasis (1 case), which can independently predict a poorer outcome, irrespective of the status of the margin clearance. Thus, additional factors like histologic subtype of tumour, MVI and metastasis prove to be factors of prognostic significance. Our results are similar to other studies by Dicken *et al*(92) and Schnater *et al*(93)who have shown that margin clearance is not an important factor in the prognostication of HB. This can be attributed to the fact that resection is carried out after a course of chemotherapy and the tumour may not be viable at the margins.

One out of the two cases with a margin of ≥1cm died of disease. This patient was found to have MEM subtype withfocal melanin pigmentation and portal vein embolus.

Immunohistochemical expression of CK19, Beta-catenin and EpCAM:

CK19 expression was found in 21.88% and 17.39% in fetal and 54.17% and 72.22% in embryonal components in the pre and post chemotherapy groups respectively. The expression of CK19 was found in 50% and 44.3% of tumours in the previous studies by Ward *et al* and Yun *et al* in the Asian and Korean population respectively(7,54). The difference between the fetal and embryonal components was statistically significant in both the pre and post chemotherapy groups in our study and this comparison was not done in the previous studies. One case with SCUD subtype also showed diffuse expression of CK19. The higher frequency of CK19 expression in the embryonal and SCUD subtypes when compared to the more mature fetal subtype confirms that CK19 is a marker of embryonic stem cell and the histiogenesis of HB from embryonic cells(94). The expression of CK19 was higher in HB (50%)when compared to HCC (33%) in various studies including a study from our institution (8,54). Although CK19 expression has been found to predict aggressive behaviour (8) and an independent factor of poor prognosis (96) in HCC, a significant difference in the survival between the CK19 positive and negative tumours in HB was not found in the study by Yun *et al* and also in our study (7).

Nuclear expression of beta-catenin expression was present in 48.65% and 57.14% of tumours in the pre and post chemotherapy groups respectively in our study. This is similar to the results of Gupta *et al*, where nuclear ± cytoplasmic beta-catenin was

seen in 61% of tumours(35). However, European studies have reported lesser expression of beta-catenin of 31-39%(86,96).

The intensity of staining of beta-catenin was strong in approximately 80% of tumours in both the pre and post-chemotherapy groups in our study but this could not be compared between the two groups due to small sample size. However Sarangarajan *et al*, have found no significant difference in the staining intensity between the pre and post-chemotherapy tumours (47).There was no significant correlation between the histological subtypes and beta-catenin expression which was similar toother studies (35, 47).

Comparison of beta-catenin expression in HB with outcome is highly variable with different studies showing different results. In our study, tumours with nuclear expression of beta-catenin had decreased EFS (45.07 months) when compared to those patients who had no expression(66.69 months), though it was not statistically significant. This is in contrast to the results of Gupta *et al* where a relatively better prognosis was seen in tumours with nuclear beta-catenin (35).

However, other studies from Europe (97) and the United States (47) have found no prognostic difference with respect to beta-catenin expression. These differences in the expression of beta catenin in different populations could be explained based on the different genetic and environmental factors and also the multiple oncogenic pathways involving beta-catenin.

EpCAM expression was seen in all tumours (100%) in the pre-chemotherapy group and about 82.14% (23 of 28 tumours) in the post-chemotherapy group and >80% of

tumours in both the groups had strong expression according to the Spizzo's grading (71). Other studies have reported EpCAM expression in 70-82%of HB, but have not described the staining intensity(52,54).The intensity of EpCAM expression was found to have an inverse relationship with the differentiation of the tumour (52). The SCUD subtype which originates from the hepatoblasts at an early stage of maturation should have the maximum expression of EpCAM. However, one tumour with SCUD subtypein our study did not show expression of EpCAM. We did not find a correlation between the epithelial subtype and intensity of EpCAM expression in our study. However, >90% of cases with strong expression of EpCAM had ≥50% viable tumour following chemotherapy and this was statistically significant. To our knowledge, there is no data available in the literature which correlates EpCAM expression and viability of the tumour.

Another interesting finding in our study was the identification of the residual viable tumour cells by EpCAM, which were initially thought to be non-neoplastic hepatocytes on the H&E section. However, EpCAM highlighted these viable clusters, thereby identifying them as residual tumour clusters, as normal hepatocytes do not express EpCAM (10). EpCAM expression would thus be useful to identify those tumour cells that mimic the normal hepatocytes, especially in post-chemotherapy tumours where maturation of tumour cells is known to occur. In addition identifying EpCAM positive tumours may aid in targeted therapy with monoclonal antibodies which would improve the survival of those patients who are resistant to conventional chemotherapy (98).

Outcome and survival:

Recurrence of tumour was found in 3 of 55 (5.45%) cases in our study and all three patients had persistent increase in AFP levels following chemotherapy/surgery. Distant metastasis was seen in 10 of 55 (18.18%) cases of which the most common site was the lung. Lower EFS and overall survival (OS) for patients who present with lung metastasis has been documented by Wanaguru *et al* and Perlingo *et al*(66,98). In our study, the OS for patients who presented with lung metastasis (n=4) was only 28.38 months when compared to 75.86 months in patients without lung metastasis (n=19), though this was not statistically significant. Wanaguru *et al* have also shown that the 2-year EFS for patients with lung metastasis was 62.5%, when compared to 89.3% in patients without lung metastasis (66).

Details about the death was available for 35 of 55 cases, of which 15 (42.85%) died of disease. This was higher than what was observed in the North Indian and the German studies, which documented only 21% and 23% deaths respectively in HB patients(35,60) and this may be due to more number of patients presenting at a higher stage of the disease in our study. However, the stage of the disease was not mentioned in the previous studies.

The overall median survival of our patients was 70.71 months, and the 5 year OS is 60% which is similar to the other studies by Allan et al (99) and Liu et al (100) who have reported a 5 year OS of 63% and 58.7% respectively. The mean EFS in our study was 59.49months and at 9 years of follow-up, the estimated freedom from metastases

was 56.5% (95% confidence interval, 34.5%-76.8%), when compared to 77% metastasis free survival at 2 years of follow up in a study by Wang *et al* (101).

Although stage of the tumour at diagnosis remains the key factor in determining the prognosis(1,31,56,91), various clinical and histological factors have also been implicated in the prognostication of HB patients in different studies. These include age at diagnosis (7), AFP level(102), multifocality and PRETEXT score(61,102), mitosis (35), histological subtype (7,101)etc. In this study, multifocality and the percentage of viable tumour ≥50% were significant factors when EFS was compared. Though not statistically significant, other factors like age at diagnosis ≤2yrs, male sex, AFP level <10000 IU/ml following chemotherapy, tumour dimension ≤5cm, PRETEXT I&II, mitosis ≤2/10hpf and absent nuclear expression of beta-catenin were found to be associated with a higher EFS rate.

In conclusion, this study has described the histopathological features and the immunohistochemical expression of three markers CK19, Beta-catenin and EpCAM in various subtypes of HB and compared with parameters of tumour behaviour and survival. Larger multicentric collaborative studies are required for proper validation of these resultsand to identify potential biomarkers which can be useful in the prognostication and treatment of HB.

CONCLUSIONS

CONCLUSIONS

- This study included a total of 55 cases of hepatobastoma, including pre(44) and post-chemotherapy (33) groups.
- The average age of the patients at presentation was 2.3 years and 78% presented within two years of life.
- The boys outnumbered the girls with a male to female ratio of 2.1:1.
- At presentation, only one patient with SCUD subtype hadnormal AFP level.
- Significant decrease in AFP was observed following chemotherapy ($p=<0.001$). Twenty one percent of cases had persistent rise in AFP following chemotherapy and were associated with recurrence, metastasis or resistant disease.
- Two out of three patients with elevated beta- hCG had a poor prognosis.
- More than 50% of the patients in this study belonged to PRETEXT II.
- Patients in PRETEXT III and PRETEXT IV succumbed to the disease (83.33% and 100%) more often when compared to those in PRETEXT I and PRETEXT II (66.66% and 7.69%), which was statistically significant ($p= <0.001$).
- Most common location of the tumour was the right lobe (57%).
- Eighty one percent of the tumours were solitary.
- Multifocality was associated with a lower EFS when compared to solitary tumours ($p=0.001$).

- There was a statistically significant difference in the size of the tumour in the pre and post chemotherapy groups (p=<0.001).
- Most common epithelial subtype was fetal and mixed epithelial in the pre and post-chemotherapy groups respectively.
- In the post-chemotherapy group, the fetal component of the tumour had mitosis of ≤5/10hpf, when compared to 47% of embryonal component (p=<0.001).
- Fetal subtype was commonly associated with steatosis when compared to embryonal and SCUD subtypes in the pre-chemotherapy group (p=0.03).
- Tumours with a predominantly fetal subtype had comparatively decreased chance of MVI, recurrence, metastasis and death.
- SCUD subtype when present even as a focal component affects outcome.
- Two patients with extensive ossification of tumour in the post-chemotherapy group had no evidence of death, recurrence or metastasis at two years of follow up.
- MVI was noted in 42% tumours in this study and was associated with lower EFS (p=0.08).
- Nineteen (57.58%) patients in this study had ≥50% viable tumour and lesser EFS when compared to patients with <50% viable tumour (p=0.04).
- Six (33.33%) cases with margin ≤0.5cm died of disease and additional factors like histologic subtype of tumour, MVI and metastasis prove to be factors of prognostic significance, irrespective of the margin clearance.
- Distant metastasis was seen in 18.18% cases of which the most common site was the lung.

- The OS for patients who presented with lung metastasis were lower (28.38 months) when compared to those without lung metastasis (75.86 months).
- CK19 expression was found most commonly in the embryonal (54.17% and 72.22% in the pre and post-chemotherapy groups) component and the one case with SCUD subtype.
- Nuclear expression of beta-catenin was seen in around 50% of tumours in this study and was associated with lower EFS when compared to those without nuclear expression.
- EpCAM expression was seen in 100% and 82% of tumours in the pre and post-chemotherapy groups respectively and strong expression of EpCAM was associated with ≥50% viable tumour following chemotherapy (p=0.04).
- EpCAM helps in the identification of residual mature tumour cells following chemotherapy, which can mimic normal hepatocytes.
- Fifteen (43%)of our patients died of disease.
- The overall median survival was 70.71 months with a 5 year OS of 60%.
- The EFS of patients in this study was 59.49 months and 56.5% of patients were metastasis free at 9 years of follow up.
- Factors like age at diagnosis ≤2yrs, male sex, AFP level <10000 IU/ml following chemotherapy, tumour dimension ≤5cm, PRETEXT I&II, mitosis ≤2/10hpf and absent nuclear expression of beta-catenin predicted a higher EFS rate.

LIMITATIONS

LIMITATIONS

Though this study has shown correlation of histological subtypes with expression of various immunohistochemical markers and tumour behavior, statistical significance could not be achieved in all parameters due to small sample size in various subgroups.

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ANNEXURES

ANNEXURES

Appendix I

Protocol for automated immunostaining:

1. Paraffin embedded tissue sections were cut at 4µ thickness and floated in poly L-lysine coated slides and incubated overnight at 37˚C.
2. These slides were then treated with 4% milk solution for 10 minutes to eliminate the hydrophobic effect and give positive charge to the slides.
3. Then the slide labels were bar coded and the labeled slides were loaded in Ventana Benchmark XT autostainer (a fully automated immunostainer).
4. Individual protocols have been designed in the software attached to the machine for each marker. Specific protocols were selected according to the marker.
5. A standard protocol was used for all the markers. The steps included in this protocol were as follows:
    a. Deparaffinization
    b. Liquid coverslip application.
    c. Heat induced antigen retrieval by treating with standard CC1 solution (pH patent with the company) for one hour at 90˚C.
    d. Then the primary antibody was added and incubated for 40 minutes at 37˚C.
    e. Then the secondary antibody (Multimer) was added and incubated for 8 minutes.

f. Finally the slides were counterstained with Harris haematoxylin and incubated for 8 minutes, followed by incubation with the bluing reagent for 4 minutes.

(From antigen retrieval till counterstaining, in between every step the slides were washed with reaction buffer. The whole process is automated).

Then the slides were brought to 80% alcohol (2 changes) to remove the liquid coverslip and then dried and mounted in DPX.

Proforma

Histopathological and immunohistochemical study of Hepatoblastoma

Name: Age/Sex: Hosp no: Bx / Resection No.

Date: Bx resection interval:

Clinical presentation:

Abd distension/ Abd mass/Abd pain / Failure to thrive/ Jaundice / Fever/ Loss of weight and appetite/ Incidental

Investigations:

AFP (IU/ml): Initial: Pre-op/Post-Chemo: Post-op: Follow up:

Beta-HCG(IU/ml):

Radiology:

Tumor location:

Left lobe / right lobe / specific segment: Tumor nodules: single / multiple

Tumor size (cms): Pre-op stage: Metastasis: Present/ Absent/ NA

Chemotherapy: Yes/ No No of cycles: Duration:

Biopsy Details:

Nature of the biopsy: No of cores: Length/Core: No of sections studied:

Gross Features:

Size: Appearance: Solid/Cystic Necrosis:

Histological Features:

Epithelial subtype: Fetal/ Embryonal/ Macrotrabecular/ Small cell undifferntiated

Arrangement: Cords/ Trabeculae/ solid nests/ Primitive tubules/ Rosettes

EMH: Present/ Absent Mitotic activity/10HPF: Fetal: Embryonal:

Fatty change (within tumor): Macro / Micro / Mixed Mild / Moderate / Severe

Cholestasis: H/C/D

Mesenchyme:

Osteoid/ Bone/ Cartilage/ Fibrous stroma

Teratoid Features:

Chemotherapy induced changes:

Necrosis : Focal/Extensive Hyalinisation: Focal/Extensive

Squamous differentiation/ Keratinisation: FB giant cell reaction: Present/ Absent

Calcification: Present/ Absent Haemorrhage/ Haemosiderophages

Viablity and margins:

Microvascular invasion: Small / Medium / Large Viable tumour(Approx %)

Margins: ≤ 1cm >1cm

Final diagnosis: Pure epithelial/ MEM with teratoid/ MEM without teratoid

Immunohistochemical marker expression:

CK-19:

Subtype: Positive/negative: Percentage of cells: Intensity:

Beta-catenin:

Subtype: Pattern of staining: Percentage of cells: Intensity:

EpCAM:

Subtype: Proportion score: Intensity score: Total score: